The Sodium Potassium ATPase, Caveolin 1 and their Interaction as Potential Anti-Breast Cancer Therapeutic Targets

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A thesis submitted to the Royal College of Surgeons in Ireland for the degree of Doctor of Medicine (MD)

RCSI

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Head of Department: Prof A.D.K. Hill
DECLARATION

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree of Doctor of Medicine is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed

Dr Mark Owens

RCSI Student Number  10109692
Date  13 May 2013
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Finally, I would like to thank Breast Cancer Ireland and the Beaumont Hospital Cancer Research Fund for their generous financial support that made this research possible. In addition, the Dept of Molecular Medicine, RCSI, and the National Biophotonics and Imaging Platform, Ireland, for use of the confocal microscope, which was itself funded by the Irish Government’s Programme for Research in Third Level Institutions.
ABSTRACT

Breast cancer pathogenesis is modulated by complex signalling pathways centred
on cholesterol-enriched membrane domains termed lipid rafts. Caveolae, a
subtype of rafts, express the protein caveolin-1, which has been suggested as
both as a tumour suppressor and promoter. One protein that interacts with
caveolin-1 is the sodium potassium ATPase (Na⁺K⁺ ATPase) ion channel. Na⁺
K⁻ ATPase has recently been implicated in cell signalling via interaction with its
natural inhibitors, cardiac glycosides (CGs). Although predominantly used for
heart failure indications, CGs reportedly have anti-proliferative properties in
tumour cells. Given the importance of caveolin-1 in malignant behaviours, we
hypothesized that CGs exert anti-breast cancer effects by modulating functional
associations between Na⁺K⁺ ATPase and caveolae. We first demonstrated
concentration- and time-dependent anti-proliferative responses to the CGs
digoxin, ouabain and oleandrin in breast cell lines and primary cultures. These
effects were most pronounced in primary cell cultures, and least in ER/PR/HER2
triple-negative cells. We next showed concentration-dependent reductions in cell
migration in response to CGs, particularly in ER-positive cells. CGs arrested the
cell cycle at both G1 and S phase in ER-positive cells, with partial arrest at G1 in
ER-negative cells. This was paralleled by increased p21 expression at both gene
and protein level in ER-positive cells, and decreased p53 expression in ER-
negative cells. CGs slightly decreased Na⁺K⁺ ATPase association with caveolae
in ER-positive cells, but increased that in ER-negative cells. Knockdown of
caveolin-1 (but not the planar lipid raft marker flotillin-1) potentiated the effects
of CGs in ER-negative but not ER-positive cells. Pharmacological lipid raft
disruption abrogated the effects of CGs in ER-positive cells, while raft
augmentation did the opposite. In contrast, raft disruption did not alter CG
effects in ER-negative cells, but augmentation potentiated their effects. Finally,
we showed that ouabain transiently activated both Src and ERK in ER-negative
cells, but induced delayed Src and ERK activation in ER-positive cells. Taken
together, our results provide novel evidence that the powerful anti-proliferative
effects of CGs in breast cancer cells involve modulations of the functional
association between Na⁺K⁺ ATPase and caveolin-1. We suggest that the Na⁺K⁺
ATPase, caveolin-1 and their interaction in caveolae represent exciting targets
for consideration in future breast cancer drug development strategies.
Chapter 1

Introduction
1.1. The Breast: Structure and Function

The human breast is a glandular structure lying bilaterally on the anterior chest wall that primarily functions to produce milk. It is dome-shaped with an axillary tail, and overlies the pectoralis major and part of the serratus anterior and external oblique muscles, the rectus sheath and costal cartilages. The breast primarily consists of adipose tissue and glandular tissue (1).

Functionally, each breast is divided into 15-25 independent pyramidal lobes, each consisting of several lobules, which in turn consist of multiple acini. The basic functional unit of the breast is the terminal duct lobular unit (TDLU), made up of several glandular acini and their draining duct. These ducts drain sequentially into subsegmental and segmental ducts, each lobule draining into their own dilated lactiferous sinus and collecting duct, eventually opening onto the nipple. These functional elements are embedded in and supported by connective tissue, which forms intralobular septa and extends into the suspensory ligaments of Cooper that attach the mammary gland to the overlying skin. A further layer of fatty tissue, the panalculus adiposus, surrounds the gland and forms the contour of the breast. It is in this layer that the vascular supply of the breast, as well as nerves and lymphatics, are distributed (2).

The arterial supply of the breast is complex, with no single source. Medial and deep parts of the breast are supplied by perforating branches of the internal thoracic artery; the lateral aspect is supplied by the lateral thoracic artery; while branches of the posterior intercostal and thoracoacromial arteries additionally...
supply the deeper aspects. Venous drainage, important for the haematogenous spread of breast cancer, is to the lateral thoracic (and in turn the axillary), internal thoracic and intercostal veins. Lymphatic drainage of the breast is also complex, and plays a vital role in metastasis. Cutaneous and subareolar lymphatic plexuses drain primarily to the axillary lymph nodes (approximately 75% of lymph); with additional drainage to the parasternal or inferior phrenic lymph nodes, or to the opposite breast (1, 2).

The size, shape and structure of the breast are extremely dynamic, and change throughout life under hormonal influences. Prior to puberty, the male and female breast are virtually identical. During puberty, however, the female breast enlarges dramatically to achieve its adult size and shape. Oestradiol secreted by the ovary stimulates growth and branching of the ducts, while the TDLU develops in response to progesterone secreted by the corpus luteum after ovulation. An increase in the supporting connective tissue adds to the development of the mature breast. Hormonal fluctuations throughout the menstrual cycle are reflected in the adult breast; with the increase in circulating oestrogens prior to ovulation resulting in proliferation and branching of the ducts; while progesterone secreted in the luteal phase causes connective tissue hyperplasia, increased blood flow and alveolar oedema; and prolactin stimulates further enlargement and secretion. These changes are reversed at the end of each menstrual cycle (3).

During pregnancy the breast is subject to stimulation by a range of hormones, including oestrogens, progesterone, prolactin, human chorionic
somatomammotropin, thyroid hormone and corticosteroids. In the early stages, epithelial proliferation results in an increase in the number of alveoli and lobules with further ductal development; while there is accompanying venous engorgement and enlargement of the areola. In the second half of gestation, progesterone stimulates an increase in vascularity, in addition to further differentiation of lobules and eventual secretion of colostrum, a thin fluid rich in protein (especially immunoglobulin) that precedes true milk secretion (2, 3).

Prolactin secretion from the maternal pituitary gland increases throughout pregnancy and remains elevated for several weeks post-partum. Its activity, however, is suppressed by high levels of circulating oestrogens and progesterone. Parturition results in a dramatic fall in circulating oestrogens and progesterone, allowing true milk secretion to occur. While prolactin levels drop to near normal levels, suckling induces a temporary prolactin surge, and can maintain milk secretion indefinitely. Suckling induces the milk letdown reflex via release of oxytocin from the posterior pituitary gland. Oxytocin in turn induces contraction of myoepithelial cells surrounding the acini and contraction of smooth muscle around the milk duct, which together result in expulsion of milk into the lactiferous sinuses. Eventual cessation of suckling causes a drop in prolactin, allowing the breast to involute to its normal state (2-4).
1.2. Aetiology of Breast Cancer

Although the aetiology of breast cancer is largely unknown, several risk factors have been identified. Up to 10% of breast cancers are related to inherited genetic mutations, with a woman’s lifetime risk increased if any first-degree relatives developed breast cancer, particularly at an early age. Approximately 50% of hereditary breast cancers are associated with mutations in the BRCA1 gene on chromosome 17q21.3 (5). Women with mutations in this gene have a 57% risk of developing breast cancer by the age of 70, in addition to an increased risk of ovarian cancer (6). A further third have mutations in BRCA2 on chromosome 13q12-13, which confers a 49% breast cancer risk, in addition to increased rates of several malignancies including ovarian, pancreatic and gastric carcinoma (5, 6). Both the above genes play a poorly understood role in DNA repair. Other inherited disorders associated with breast cancer include Li Fraumeni syndrome (caused by mutations in TP 53) and Cowden disease (caused by PTEN mutations) (5, 7, 8). In addition to genetic factors, further risk factors include:

- Lifelong exposure to high levels of oestrogen (nulliparity & low parity, early menarche, late menopause, late first pregnancy (8, 9), oral contraceptives, hormone replacement therapy, diethylstilbestrol exposure (10))
- Smoking (11)
- Alcohol consumption (11, 12)
- Age: highest incidence in 50-64 age group
- Radiation exposure (environmental, previous radiotherapy)
• Previous breast disease (previous breast cancer, ductal carcinoma in situ [DCIS], benign breast disease)
• High income socio-economic group (13)
• Sedentary lifestyle (10)

Factors that reduce the risk of developing breast cancer include physical activity, breastfeeding, diet (dairy, fibre, vitamin D, low red meat intake). Interestingly, a greater amount of body fat appears to be protective against pre-menopausal, but is a risk factor for post menopausal, breast cancer (14-17).
1.3. Breast Cancer Statistics

Worldwide, over 1.2 million women are diagnosed with breast cancer annually. Breast cancer is currently the 2\textsuperscript{nd} most prevalent cancer among Irish women. It represents 32.3\% of all invasive cancers other than non-melanoma skin cancer in this group, with an average of 2673 new cases diagnosed per year from 2007-09, and a total lifetime risk of 10\% (18). However Irish mortality from the disease has declined by 28\% from 1994 to 2009 (19). Ireland had the fourth-highest incidence and mortality from female breast cancer in the EU in 2008 (20), and the incidence of new breast cancers has increased by on average 1.9\% per year from 1994 to 2009 (19).

The overall relative 5-year survival in Ireland was 81.4\% for those diagnosed between 2004-08, compared to 71.8\% for those diagnosed between 1994-98. Breast cancer 5-year survival in Ireland from 2000-02 was the 5\textsuperscript{th} lowest among countries in the EU with national cancer registries, and was below the Eurocare mean of 79.0\% (13). Comparison figures since the introduction of countrywide screening in Ireland are not yet available.

Although over 80\% of women now present with operable disease (13), it is estimated that approx 50\% of these will experience at least one relapse. While the majority of presentations are in women aged over 50 (73\% in Ireland) (13), those cases that do occur in younger women are often associated with a more aggressive phenotype. As expected, the national breast cancer screening programme has resulted in a relative increase in the cancers diagnosed in the 50-
64 years age group. The median age at diagnosis is 59 (21), while that at death is 72 (22).

1.4. Pathology and Classification of Breast Cancer

While breast cancers are heterogenous, the vast majority arise from the terminal duct lobular unit of the breast and are thus classified as mammary adenocarcinomas. There are several classification systems, based on histological appearance, hormone receptor status, grade, stage, genetics and molecular subtypes; which will be discussed in greater detail in the following paragraphs.

1.4.1 Histological Classification

Broadly speaking, breast cancer can be divided into invasive carcinoma (carcinoma that has penetrated the basement membrane) or noninvasive/carcinoma in situ (those that have not). Carcinoma in situ can be further subdivided into ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS). DCIS is so named as it forms in and is confined to milk ducts. It may exhibit a variety of architectural forms, including solid, micropapillary, cribriform, clinging and comedo subtypes (5). They may also be classified by nuclear grade into high, intermediate and low. Approximately a third of women
with untreated DCIS develop invasive carcinoma, which generally occurs in the affected area, and risk is directly proportional to nuclear grade (5, 23).

LCIS displays a characteristic uniform appearance, with monomorphic cells in the ducts and lobules. Unlike DCIS, it rarely forms palpable masses and is almost always an incidental finding on mammography or biopsy. LCIS is commonly multifocal or bilateral. It most likely constitutes a marker for increased risk of invasive carcinoma rather than a direct precursor, as women with LCIS have an equal risk of developing invasive carcinoma in either breast (24).

Invasive carcinoma can also be further subdivided into invasive ductal carcinoma (IDC), invasive lobular carcinoma, cribriform, inflammatory, tubular, mucinous and medullary carcinomata. The most common histological type is invasive ductal carcinoma (75%). This term implies that there are no "special type" histological features present. It tends to present as a hard discrete mass. The microscopic features vary from well-differentiated types with tubule and gland formation through to anaplastic forms. Margins are usually irregular, and this subtype tends to be associated with DCIS. Approximately two thirds express oestrogen and progesterone receptors, while one third overexpress human epidermal growth factor receptor 2 (HER2) (5).

Invasive lobular carcinoma is the 2nd most prevalent histological type, and is associated with LCIS, consisting of cells with identical morphology. They are frequently multicentric or bilateral, and generally have a better prognosis than
IDC. They have a tendency to metastasise to serosal surfaces. Terminal ducts and lobules are filled with malignant cells, which may invade individually or in chains into the stroma. They nearly always express hormone receptors, while HER2 overexpression is extremely rare (5).

Inflammatory carcinoma implies a clinical rather than histological appearance of a diffusely enlarged inflamed breast due to obstruction of lymphatic drainage by tumour cells and carries a poor prognosis. Tubular carcinoma is a subtype that tends to form small tumours with low metastatic potential, consisting of well-differentiated tubules. Mucinous or colloid carcinomas are commonly gelatinous tumours, characterised histologically by expression of extracellular mucin. Medullary carcinomas grossly resemble fibroadenomata, but histologically consist of high grade anaplastic cells. They are more common in patients with BRCA 1 mutations, and tend to lack hormone receptors (5).

1.4.2. Breast Cancer Staging

Breast cancer is commonly staged by the tumour node metastasis (TNM) system. These American Joint Committee on Cancer Classification guidelines describe breast cancer using a combination of primary tumour size, nodal status and presence or absence of distal metastases.
<table>
<thead>
<tr>
<th>Table 1.1. Breast cancer TNM staging (25)</th>
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<table>
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<tr>
<th>Primary Tumour (T)</th>
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<table>
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<tr>
<th>Regional Lymph Nodes (N)</th>
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<th>Metastases (M)</th>
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<td>cM0(++)</td>
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<tr>
<td>M1</td>
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</tbody>
</table>
This clinico-pathological staging system can be further classified into prognostic stages I-IV.

Table 1.2. Anatomic stage and prognostic groups (25)

<table>
<thead>
<tr>
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<th>M0</th>
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<td>N0</td>
<td>M0</td>
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<tr>
<td>Stage IB</td>
<td>T0</td>
<td>N1mi</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>N1mi</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIA</td>
<td>T0</td>
<td>N1</td>
<td>M0</td>
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<tr>
<td></td>
<td>T1</td>
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<tr>
<td>Stage IIB</td>
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<td>T3</td>
<td>N0</td>
<td>M0</td>
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<tr>
<td>Stage IIIA</td>
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<td>Any T</td>
<td>N3</td>
<td>M0</td>
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<tr>
<td>Stage IV</td>
<td>Any T</td>
<td>Any N</td>
<td>M1</td>
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</tbody>
</table>

5 year survival rates are closely related to stage, ranging from 92% for stage 0 to 13% for stage IV disease (5).
1.4.3. Grading of Breast Cancer

Breast cancer can also be described in terms of histological grade, a measure of its degree of differentiation, using the Elston-Ellis modification of the Bloom-Richardson grading system. This uses the three parameters of tubule formation, nuclear pleomorphism and mitotic activity to give an aggregate score of between three and nine. Tubule or duct formation in the whole tumour is scored from one (tubule formation in over 75% of tumour) to three (tubules in less than 10%).

Nuclear pleomorphism or variation is scored from one (minimal variation in size and shape) to three (marked variation). Mitotic index, or number of mitotic figures at the tumour margin per ten microscope fields, is a marker of proliferation, and is similarly scored from one (0-9 mitotic figures) to three (over 19 mitotic figures).

An aggregate score of 3-5 indicates grade 1, or well-differentiated. A score of 6-7 indicated grade 2 (moderately-differentiated), while 8-9 is grade 3 (poorly-differentiated). This grading system is second only to nodal status as a prognostic indicator (26, 27).

1.4.4. Receptor Status

The presence or absence of oestrogen receptor (ER) and progesterone receptor (PR) on tumour tissue is important, not only as an indicator of differentiation, but also as an indicator of potential susceptibility to anti-oestrogen therapies. Tumours expressing both receptors have a high response rate (~85%). Tumours
expressing only one of the two have a lower response rate (25-45%), while absence of both receptors indicates the lowest response rate (≈10%) (5).

Androgen receptor is expressed by 80-90% of ER-positive tumours and 40% of ER-negative (28). While its stimulation inhibits growth in ER-positive tumours, it may have the opposite effect in ER-negative cancers, and its expression may be utilized as a prognostic marker and therapeutic target in the future (28-30).

Overexpression of HER2 occurs in approximately 30% of breast cancers, and has been associated with a worse prognosis in the past. However, the monoclonal antibody Trastuzumab/Herceptin specifically downregulates tumorigenic signalling from this receptor, significantly improving the outcome of HER 2-overexpressing tumours (31).

1.4.5. Molecular Subtype

More recently, a classification of breast cancer using a combination of genetic profiling and histological grade is increasingly being utilised, which divides breast cancer into several molecular subtypes; luminal A, luminal B, basal-like, HER2- overexpressing, normal breast-like; and more recently claudin-low (32, 33).

Luminal A is the most common subtype, accounting for 50-60% of breast cancers. It is characterized by high expression of genes normally associated with mammary duct luminal epithelium, high levels of ER- and PR-positivity, low levels of HER2 positivity, low expression of proliferative genes and low
histological grade. This subtype includes all LCIS, as well as most invasive lobular carcinomas, and generally carries an excellent prognosis. Relapse rate is lower than other subtypes, and metastasis occurs most commonly to bone (34, 35).

Luminal B accounts for 10-20% of breast cancers. Like luminal A, this subgroup tends to express ER and PR and be HER2 negative, but tends to be more aggressive, with higher histological grade and increased expression of proliferative genes such as Ki67. It carries a worse prognosis than luminal A cancers (34). While these tumours also most commonly metastasize to bone, they have a higher incidence of spread to the liver (35). They have a relatively higher response rate to neoadjuvant chemotherapy than luminal A cancers.

The basal-like subtype accounts for 10-20% of tumours and is so named as these cancers tend to express genes associated with myoepithelial cells, such as CK 5 and EGFR, and have a relatively lower level of expression of luminal genes. They are most commonly invasive ductal carcinomas that lack ER, PR and HER2 and are therefore termed triple negative. They tend to be aggressive, presenting as large high-grade tumours, often in younger women and those of African origin. They metastasize early, more commonly to lung, central nervous system and lymph nodes (35). They have a high rate of p53 mutations and are often associated with BRCA1 mutations. Although more chemosensitive than luminal tumours, they have a higher relapse rate and an overall poorer prognosis (34).
The HER2-enriched subtype includes 15-20% of tumours. Despite the name, only 70% of these tumours overexpress HER2, and not all HER2-overexpressing tumours fall into this molecular subgroup. While these tumours do not express basal-like genes, they may exhibit low-level expression of luminal genes. The majority of these tumours are highly proliferative, ER- and PR -negative, with a high histological grade and a high incidence of p53 mutations. This group has been further subdivided into three distinct subgroups, of which one group carries a much worse long term survival (36). Overall, HER2-enriched tumours are initially more chemosensitive than luminal tumours, but carry a poorer overall prognosis, although the development of anti-HER2 targeted therapies has improved the prognosis in recent years (31).

The normal breast-like subtype represents 5-10% of cancers and is poorly characterized, generally being triple-negative but lacking basal type genes and expressing some genes characteristic of adipose tissue. They are usually poorly responsive to chemotherapy, and have a prognosis intermediate between luminal and basal-like tumours. Some authors doubt the real existence of this subtype, suggesting that they may in fact represent technical artefact due to contamination with normal tissue (37).

The claudin-low subtype was described more recently than the others, and is so named due to its low expression of cell-cell adhesion proteins including claudins (33). It accounts for 12-14% of breast cancers. These tumours have some similarities with the basal subtype, with both groups exhibiting low luminal gene expression and most commonly being triple negative. In contrast to the basal
subtype, claudin-low tumours overexpress several genes relating to the immune response, as well as a number of mesenchymal genes. They are commonly high-grade ductal carcinomas, but may also exhibit medullary or metaplastic differentiation, and may be associated with BRCA1 mutations. Their chemosensitivity is intermediate between that of luminal and basal like cancers, and they carry a poor prognosis (34).
Table 1.3. Characteristics of the molecular subtypes of breast cancer (34)

<table>
<thead>
<tr>
<th>Subtype</th>
<th>ER/PR/HER2</th>
<th>CK 5/6, EGFR</th>
<th>Genes Expressed</th>
<th>Grade</th>
<th>Proliferative Genes</th>
<th>P53</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A</td>
<td>ER+ PR+</td>
<td>-</td>
<td>ESR1, GATA3, KRT8, KRT18, XBP1, FOXA1, TFF3, CCND1, LIV1</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Luminal B</td>
<td>ER +/- PR +/-</td>
<td>-</td>
<td>ESR1, GATA3, KRT8, KRT18, XBP1, FOXA1, TFF3, SQUIE, LAPTMB4</td>
<td>Intermediate</td>
<td>High</td>
<td>Intermediate</td>
</tr>
<tr>
<td>Basal-Like</td>
<td>ER- PR-</td>
<td>+</td>
<td>KRT5, CDH3, ID4, FABP7, KRT17, TRIM29, LAMC2</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>HER2- Enriched</td>
<td>ER- PR-</td>
<td>+/-</td>
<td>ERBB2, GRB7</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Normal Breast-Like</td>
<td>ER-/+</td>
<td>+</td>
<td>PTN, CD36, FABP4, AQP7, ITGA7</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Claudin-Low</td>
<td>ER- PR-</td>
<td>+/-</td>
<td>CD44, SNAI3</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
</tbody>
</table>
Fig. 1.1. Relative expression of oestrogen receptor (ER) and HER2 by molecular subtype (adapted from (34)).

Even newer evidence in the form of a 2012 paper has since emerged indicating that analysis based on both gene expression profiles and copy number aberrations reveals even more heterogeneity than previously believed, with the addition of at least ten new subgroups (38). It is quite likely that this number will be further increased in the coming years with the discovery of new prognostic markers.
1.5. Clinical Presentation and Diagnosis of Breast Cancer

Clinical presentation of breast cancer is quite variable. Breast cancer may involve any portion of the breast, including the axillary tail, although it occurs most commonly in the upper outer quadrant. The most common presentation of breast cancer is as a hard mass, which may become fixed to the overlying and surrounding tissues with local progression. Retraction of involved suspensory ligaments of Cooper may lead to retraction and elevation of the breast with complete or partial inversion of the nipple. Further abnormalities involving the nipple may include nipple discharge or Paget's disease, a condition that mimics eczema of the nipple and areola secondary to cutaneous involvement. Skin involvement may manifest as a dimpled appearance known as peau d'orange, while further progression and local invasion may result in the diffuse erythematous appearance of inflammatory carcinoma, or ulceration of the overlying skin (39).

Breast cancer is routinely diagnosed by triple assessment; a combination of thorough history and clinical examination, imaging and pathological examination. Mammography, or plain X-ray of the breast, is the preferred imaging modality in the majority of women, and utilizes low dose ionizing radiation to detect suspicious calcifications as small as 100\(\mu\)m in diameter. The majority of malignancies are detectable on mammography at least a year before a mass is palpable. In younger women, however, increased breast tissue density renders mammography less sensitive, and in these patients ultrasonography (US) is the investigation of choice for examining lesions detected by physical
examination. It is also useful in differentiating cystic and solid masses, for examination of axillary lymph nodes and for guiding percutaneous biopsy of lesions (39).

Other imaging modalities are also frequently used in the staging of established cancer. While in patients with small tumours a plain chest X-ray is sufficient, those with locally advanced tumours will require more extensive imaging. This may include computed tomography (CT) of the thorax and abdomen and/or brain to detect visceral metastases. This investigation uses ionizing radiation to obtain three-dimensional images of the area of interest. Bone scintigraphy is a nuclear medicine investigation used to diagnose bony metastases. The patient is injected with a radioisotope such as Technetium-99m medronic acid, which preferentially localizes in areas of high bone turnover. The emitted radiation is detected by scanning with a gamma camera. Positron emission tomography (PET), often in combination with CT (PET-CT), is another nuclear medicine investigation that utilizes uptake of $^{18}$F-fluorodeoxyglucose, a positron-emitting glucose analogue, to identify areas of increased metabolic activity in three dimensions. It can detect both primary and metastatic malignancies, regardless of location, and can thus be used both for staging and screening, but is limited by its expense. Magnetic resonance imaging (MRI) uses the phenomenon of magnetic resonance to obtain three-dimensional views, and thus avoids ionizing radiation. It is more sensitive than mammography and US, although it is less specific and tends to overestimate tumour extent (40). Its main utility probably lies in follow up of patients with previous breast-conserving surgery with reconstruction, as well as an adjunct to screening mammography and examination in selected high risk patients (41).
Biopsy of lesions is performed by fine needle aspiration cytology (FNAC), in which a fine needle is used to retrieve cells from the lesion, which are then stained and examined microscopically. The usefulness of this technique may be limited by the lack of preserved histological architecture, and if histological specimens are required more invasive biopsy techniques such as core biopsy, which uses a wider bore needle, or excisional (surgical) biopsy, may be used. Small lesions may also be localized prior to surgical biopsy by insertion of wires under radiological guidance.

While in the past the majority of breast cancers presented symptomatically, the recent introduction of a national breast cancer screening programme is likely to dramatically change the pattern of breast cancer presentation. Screening was initially introduced in the USA in 1990, and was piloted in Ireland in the early 1990’s. Phase one of BreastCheck was introduced in Ireland in 2000, offering free two-yearly mammograms to women aged 50 to 64 in the East, and was gradually rolled out nationwide after this, with the last unit opening in 2007. The programme currently screens over 120,000 women annually, and has detected over 5,000 cancers since its inception (42). It is estimated that for women aged 50 to 59 undergoing biannual screening mammography, 351 women must be screened for every life saved (43).
1.6. Treatment of Breast Cancer

1.6.1. Surgery

The wide variety of modalities in use for the treatment of breast cancer reflects the heterogeneity of the disease. Surgical excision of the primary tumour with adequate normal margins remains the only curative treatment, while palliative surgery may be undertaken for symptomatic relief in advanced disease. Radical mastectomy was first performed by Halsted in 1882, and initially involved excision of the breast, axillary lymph nodes and pectoralis major and minor muscles, under the principle that a greater amount of tissue excised correlated with a better chance of cure. This operation has since been abandoned with similar oncological results being achieved with less invasive procedures and adjuvant therapies (39).

Many tumours are suitable for breast-conserving surgery, which usually entails wide local excision consisting of the tumour with at least one centimetre margin of normal tissue. Tumours that are large relative to the size of the breast, tumours that are located directly beneath or involve the nipple areola complex (NAC), recurrent or multifocal disease normally require mastectomy. Axillary surgery or sentinel lymph rode biopsy (SLNB) is commonly undertaken at the same time. The latter procedure involves injection of a radionuclide around the nipple prior to surgery, usually in addition to injection of blue dye for added specificity. The axillary lymph node(s) with maximum radionuclide uptake as measured by Geiger counter is then excised for histological examination. This is
the sentinel node, i.e. the first node through which lymph draining the tumour should pass, and the absence of metastatic disease in this node usually signifies absence of axillary nodal spread. A positive SLNB usually necessitates further axillary clearance surgery. However, in the last two years, a number of large studies have shown that further axillary surgery confers no survival advantage over SLNB-positive tumours treated with primary excision and adjuvant radiotherapy, and it is likely that axillary clearance will become increasingly rare with the exception of gross nodal disease (44, 45).

1.6.3. Adjuvant Therapy

A large proportion of women undergoing curative surgery for breast cancer will also require some form of additional therapy. Adjuvant therapy may improve survival after surgical resection by eradicating undetected micrometastases, and postoperative chemotherapy or hormone therapy improves disease-free survival by approximately 30% (46). The current modalities of adjuvant therapy include radiotherapy, hormone therapy, chemotherapy and anti-HER2 therapy, although it is likely that further targeted therapies will be routinely adopted in the future. A number of expert groups both in the USA and Europe have published guidelines for selection of adjuvant therapy for breast cancer treatment (47, 48). The 2011 St Gallen Expert Consensus report recommends the use of endocrine therapy in luminal A cancers, with the addition of chemotherapy in luminal B. Chemotherapy is recommended for other groups, with the addition of Trastuzumab in HER2-overexpressing tumours (47).
1.6.4. Radiotherapy

Ionising radiation has been used for over a century in the treatment of a variety of malignancies. It induces DNA damage in tumour cells, thus resulting in tumour necrosis. External beam radiotherapy to the remaining breast is usually administered after conservative surgery. Chest wall radiotherapy may also be administered after mastectomy in higher risk patients, such as those with large tumours, lymphovascular invasion or extensive nodal disease. Radiotherapy may also be administered to the axilla or other metastatic deposits. Side effects include local skin irritation and an increased risk of secondary malignancy. Side effects may be reduced by delivering the total dose in several smaller fractions. This allows normal cells to repair themselves between doses, while tumour cells, which are less efficient at DNA repair, cannot do so as efficiently (49).

1.6.5. Chemotherapy

Cytotoxic chemotherapy usually employs a combination of agents. Cyclophosphamide, methotrexate and 5-fluorouracil has been the most commonly used regimen for adjuvant therapy since the 1970s, and can achieve a 34% improvement in disease-free and overall survival, with sustained improvement after 30 year follow up (50, 51). More modern regimens include anthracyclines, taxanes and alkylating agents such as cyclophosphamide. Several large trials in recent decades have consistently shown improved overall and disease-free survival with combination chemotherapy, particularly in ER-negative disease (52-54). Neoadjuvant chemotherapy may also be used prior to surgical treatment, either to facilitate breast conserving surgery (55), or to allow
potentially curative surgery. In inflammatory breast cancer, neoadjuvant anthracycline-based chemotherapy, followed by surgery and adjuvant chemoradiotherapy, results in a median survival of over two years and a five year survival of 30%, compared to 5% historically (56, 57).

1.6.6. Endocrine Therapy

The importance of oestrogen in breast cancer has long been recognised, with the earliest hormonal therapy consisting of surgical castration. Tamoxifen is currently the most widely used hormonal agent, and has been in use since the 1980s. It is a selective oestrogen receptor modulator, inhibiting oestrogen signalling in the breast but stimulating oestrogen receptors in other tissues including bone and endometrium, thus protecting against osteoporosis while slightly increasing the risk of endometrial carcinoma. It is currently the first line adjuvant treatment in premenopausal women with hormone receptor-expressing tumours, although it is also effective in postmenopausal women, and reduces mortality by 31% (58).

Aromatase inhibitors (AIs), such as anastrozole and letrozole, function by inhibiting aromatase, the hormone that synthesizes oestrogen from other steroid hormones such as androgens. This constitutes the only source of oestrogen in the postmenopausal woman, and as such AIs constitute the first line hormonal adjuvant therapy in postmenopausal breast cancer. As they have no effects on ovarian oestrogen production, they are of no benefit in premenopausal breast cancer. Several trials have demonstrated an improvement in disease-free
survival in postmenopausal breast cancer treated with AIs compared to tamoxifen (59, 60).

1.6.7. Targeted Therapy
While the treatment modalities described above are relatively non-specific (with the exception of hormone-based therapies); it is likely that the future of cancer therapy will lie in small molecule inhibitors of various aspects of pro-malignant signalling pathways. While such therapies will require more detailed tumour characterisation than is currently the clinical norm, they will allow development of bespoke patient-centred cancer treatment.

Trastuzumab is a monoclonal antibody against the HER2 receptor that was approved by the FDA in 1998, and is currently the most widely used targeted therapy for breast cancer. Results of five randomised controlled trials have shown an overall improvement in disease-free survival of around 50%, with a 33% improvement in overall survival with the addition of trastuzumab to adjuvant chemotherapy (61-64). Lapatanib is a new agent that inhibits both HER2 and epidermal growth factor receptor (EGFR), and trials are currently in progress examining the effects of dual anti-HER2 therapy.

Several other targeted therapies have shown promising results in clinical trials, but have not yet come into routine clinical use. These include vascular endothelial growth factor (VEGF) receptor inhibitors (65); insulin-like growth factor 1 receptor inhibitors (66, 67); polyadenosine diphosphate ribose polymerase polymerase (PARP) inhibitors (68); Src kinase inhibitors (69); and
inhibitors of the PI3K/Akt/mTor pathway (70). It is likely that targeted therapies will eventually allow the development of personalised breast cancer treatment for the majority of patients.

1.7. Pathophysiology of Breast Cancer

As this thesis aims to examine potential targets for anti-breast cancer treatments at the subcellular level, it is vital to first have an understanding of some of the complex cellular and subcellular mechanisms whose dysregulation underlies much of the observed neoplastic behaviours of breast cancer.

1.7.1. The Cell Cycle and Mitosis

The cell cycle is a continuous cycle of nucleic acid synthesis and cellular growth and division, under the control of a complex system of positive and negative regulators that exert their influence at a number of checkpoints within the cycle. It is divided into a number of distinct phases.

- **G₀ Phase**, also known as the quiescent or resting phase, is a phase where the cell is officially outside the cell cycle. It is distinct from senescence in that G₀ cells remain viable and can potentially re-enter the cell cycle at S phase. Certain cells, such as neurons or cardiac myocytes, cease replication on reaching maturity and enter a prolonged G₀ phase.

- **G1 Phase**, also known as the post-mitotic phase, is a variable period of cell growth incorporating protein and organelle synthesis. Cyclin D and E and the cyclin-dependent kinases (see below) CDK 2, 4 and 6 are
active during this phase. At the end of G1, cells must pass through the restriction point, the first major checkpoint in the cycle, after which they either pass into S phase or revert to quiescence. Transition through the restriction point is driven by cyclin E-CDK 2 complexes (71). Serum starvation results in cell cycle arrest at this point (72).

- The S, or synthesis phase, primarily involves DNA replication. During this phase, the DNA content doubles, although the total number of chromosomes remains unchanged, and this forms the basis of flow cytometric cell cycle analysis (see below). Cyclin A-CDK 2 complexes both initiate DNA replication and prevent re-replication. The S phase checkpoint is less well understood than other checkpoints, but is believed to be mediated by the protein claspin (73).

- G2 phase, as its name suggests, is a second period of cell growth and protein synthesis and preparation for mitosis. Cyclin A-CDK 1 complexes drive progression into M phase. The G2/M transition checkpoint arrests the cell cycle at this point in response to DNA damage, via both p53-dependent and -independent mechanisms (74).

- M phase includes mitosis, which is further divided into four subdivisions, and cytokinesis.
  - Prophase, in which chromatin condenses into individual chromosomes, while microtubules form projecting from the two centrosomes into the nucleus, constituting the mitotic spindle.
- In prometaphase and metaphase, the chromosomes line up between the two centromeres, and microtubules attach to each chromatid. The spindle assembly checkpoint prevents further progression until all chromosomes are aligned and attached to the spindle (75).

- During anaphase, chromosomes are cleaved into two chromatids, which are pulled apart by the shortening spindle. In addition, the centromeres further separate and the cell lengthens.

- In telophase, the cell continues to elongate, while nuclear membranes form around the daughter chromosomes at either end, which decondense into chromatin.

Cytokinesis is not part of mitosis but is the final event of M phase, whereby a cleft known as the cleavage furrow forms, dividing the cell into two daughter cells (76).

Progression through M phase is driven by Cyclin B-CDK 1 complexes.

### 1.7.1.2. Regulation of the Cell Cycle

Several proteins regulate the cell cycle. The cyclins are a group of proteins that exert their effects on the cell cycle via their interaction with cyclin-dependent kinases (CDKs). Expression of the various cyclins typically oscillates throughout the cell cycle, with the exception of G1 phase cyclins such as cyclin D, whose levels increase steadily throughout the cycle (77). In contrast, expression of CDKs is relatively constant, although their activity fluctuates with cyclin expression. CDKs form complexes with cyclins, and are then targeted to specific subcellular locations and activated, in turn phosphorylating a range of downstream effectors. CDK-activating kinase (CAK), which phosphorylates and
activates several CDKs, is itself a complex of cyclin H, CDK 7 and the protein MAT 1 (78).

<table>
<thead>
<tr>
<th>CDK</th>
<th>Cyclin</th>
<th>Peak Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDK 4</td>
<td>Cyclin D1, D2, D3</td>
<td>G₁ Phase</td>
</tr>
<tr>
<td>CDK 6</td>
<td>Cyclin D1, D2, D3</td>
<td>G₁ Phase</td>
</tr>
<tr>
<td>CDK 2</td>
<td>Cyclin E</td>
<td>G₁/S Transition</td>
</tr>
<tr>
<td>CDK 2</td>
<td>Cyclin A</td>
<td>S Phase</td>
</tr>
<tr>
<td>CDK 1</td>
<td>Cyclin A</td>
<td>G₂/M Transition</td>
</tr>
<tr>
<td>CDK 1</td>
<td>Cyclin B</td>
<td>Mitosis</td>
</tr>
<tr>
<td>CDK 7</td>
<td>Cyclin H</td>
<td>All phases (CAK)</td>
</tr>
</tbody>
</table>

Table 3.1. Cyclins and Cyclin Dependent Kinases (CDKs), and their activity within the cell cycle (after Vermeulen et al (79))

CDKs can be inactivated by inhibitory phosphorylation by enzymes such as WEE 1 and Myt 1. In addition, CDK inhibitors belonging to the INK4 and Cip/Kip families can bind to and inactivate CDKs. The INK4 family, which includes the proteins p15, p16, p18 and p19, bind to CDK 4 and CDK 6 to prevent cyclin D binding (80). The Cip/Kip proteins, which include p21 (Cip1), p27 (Cip2) and p57 (Kip2), bind to and inactivate G1 phase CDK-cyclin complexes (81). In addition, p21 directly inhibits DNA synthesis via its action on proliferating cell nuclear antigen (PCNA) (82).
The tumour suppressor p53 is one of the most important negative regulators of the cell cycle. It is phosphorylated by protein kinases in response to DNA damage, thus preventing its degradation. Protein p53 stimulates the transcription of several genes, including p21, arresting the cell cycle at the G1/S phase transition (83). p53 also stimulates DNA repair mechanisms, and stimulates apoptosis in the presence of irreparable DNA damage (84). The significance of p53 in DNA repair mechanisms is such that approximately 50% of cancers have been reported to exhibit p53 mutations (85).

1.7.2. The Malignant Phenotype

Cancer can essentially be considered a disease of dysregulated cell growth and proliferation. A crucial point is the loss of regulation of the cell cycle, resulting in uncontrolled cell division and abnormal growth (86). Further abnormalities include loss of polarity (87), dedifferentiation, abnormal cell-cell interactions (88), dysregulated migration and invasion (89). In this section, we will further consider some of the dysregulated processes involved in breast cancer growth and dissemination.

1.7.3. Mechanisms of Breast Cancer Spread

As the majority of cancer deaths are due to metastases rather than the primary tumour, a better understanding of the process of tumour cell dissemination is crucial to cancer research. Tumour growth and development of invasive potential is a complex progression that involves (at a minimum) dysregulation of cell proliferation, survival and migration. Breast carcinoma consists of malignant
epithelial cells suspended in stroma, itself consisting largely of connective tissue with interspersed blood vessels and inflammatory cells, and from which the tumour cells are usually separated at least in part by basement membrane. The stroma constitutes the majority of the tumour mass; over 90% in many breast cancers (90), and its role in cancer pathophysiology is now recognised to be far more complex than previously believed. For example, carcinoma-associated fibroblasts (CAFs) secrete stromal-cell-derived factor 1, which binds to and stimulates the receptor CXCR4 on tumour cells, attracting endothelial precursor cells and stimulating angiogenesis. CAFs implanted with non-tumourigenic prostate epithelial cells into mice result in tumour formation, while the converse occurs when malignant prostatic cells are implanted with normal fibroblasts (91), and culturing of breast cancer cells with CAFs promotes their growth (92).

Stromal immune cells play a dual role in cancer regulation. The adaptive immune system in the form of lymphocytes is involved in immunosurveillance of tumour cells, and a lymphocytic infiltrate is associated with a better prognosis in some cancers (93). However, T and B lymphocytes have also been observed to promote carcinogenesis in one mouse model (94). Similarly, the innate immune system in the form of macrophages, mast cells, granulocytes and monocytes, through their functions in antigen presentation and cytokine secretion, may facilitate an anti-neoplastic immune response. Paradoxically, however, they may also secrete pro-inflammatory cytokines, matrix metalloproteinases and angiogenic factors that promote matrix degradation, tumour survival and cancer progression (95, 96).
The phenomenon of epithelial-mesenchymal transformation (EMT) in the progression of carcinoma has been well-described, whereby the morphology of tumour cells progressively changes from the highly-differentiated appearance of epithelium to the more disordered mesenchymal cell morphology. Cells undergoing EMT downregulate epithelial proteins such as E-cadherin and cytokeratins, acquiring instead the expression of mesenchymal proteins such as N-cadherin and vimentin. This allows them to lose certain cell-cell and cell-matrix interactions, in turn increasing cell motility and facilitating invasion and distal metastasis. \textit{In vivo}, EMT in carcinoma may be visualised as a progressive loss of epithelial morphology progressing to a mesenchymal phenotype (97). EMT is induced by several factors produced both by tumour and stromal cells, including transforming growth factor-\(\beta\) (TGF-\(\beta\)), insulin-like growth factors (IGFs), epidermal growth factor (EGF), fibroblast growth factor (FGF), and hepatocyte growth factor (HGF) (96).

A logical progression from EMT is the facilitation of tumour cell migration. Cell migration is a repetitious cycle that occurs in many physiological processes including growth, tissue repair and immunosurveillance, as well as pathophysiological processes including invasion and metastasis. Broadly speaking, cell migration consists of five cyclical steps. It begins with the formation of protrusions known as pseudopodia at the leading edge, driven by actin polymerisation (98). These form small transient adhesions to extracellular matrix near the leading edge, which may recruit proteinases that then cleave extracellular matrix barriers (99, 100). The cell body translocates forwards driven by actin-myosin shortening (101). Release of adhesions at the rear of the
cell and retraction of the rear complete the cycle (102). Normal epithelium and highly differentiated carcinomas tend to exhibit collective migration and invasion, whereby cell-cell interactions are retained and migration occurs in single sheets or strands (103). In contrast, inflammatory cells, mesenchymally-derived tumour cells and poorly differentiated carcinoma cells with loss of strong cell-cell contacts tend to migrate individually (104).

1.7.4. Lipid Rafts & Caveolae

Underlying all the malignant behaviours described above are complex cascades of signalling molecules; many of which are centred on domains of the cell membrane known as lipid rafts. The widely accepted Singer-Nicolson model of the plasma membrane describes the membrane as a fluid bilayer consisting of phospholipids, oriented with the polar portions on the external surfaces and hydrophobic tails on the internal aspect; and with molecules such as proteins and cholesterol randomly interspersed (105). More recently, this model has been increasingly viewed as an oversimplification, and it is now widely recognised that sphingolipid, cholesterol and several proteins involved in dynamic cellular processes are segregated into more tightly-organised microdomains known as lipid rafts.

Beginning in the 1970’s, several authors postulated the existence of membrane microdomains, based on such observations as the ability of differing phases to co-exist in lipid bilayers (106, 107); differential distribution and clustering of membrane lipids (108-110); and the presence of sphingolipid-enriched detergent-resistant regions of cell membranes (111). Simons et al were among the first to
define the concept of lipid rafts (112), while Brown and Rose described a method of isolating them based on their insolubility in the detergent Triton X100 (113). While the concept and definition of lipid rafts have varied over the years, the 2006 Keystone Symposium of Lipid Rafts and Cell Function define rafts as “small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions” (114).

In addition to sterols and sphingolipids, several proteins preferentially compartmentalize in lipid rafts, particularly glycosylphosphatidylinositol (GPI)-anchored proteins, which are commonly used as experimental lipid raft markers. While lipid rafts are enriched in cholesterol and sphingolipids, they typically contain less phosphatidylcholine than the surrounding membrane.

Two broad subdivisions of lipid rafts exist, namely planar and caveolar lipid rafts (caveolae). Planar lipid rafts lack distinguishing morphological features and are continuous with the surrounding membrane; thus they can only be studied indirectly. They express one or more of the highly conserved flotillin proteins, flotillin-1 and -2 (115), whose functions are not fully understood. Unlike caveolae, they are present in neurons. Caveolae are distinctive flask-shaped invaginations in the membrane (the name originating from Latin, meaning “little caves”), readily visible by electron microscopy. They express one or more of the structural proteins, caveolin-1, -2 and -3.
Caveolin-1 is the most ubiquitous caveolin. A 22 kDa protein, it acts as the main structural component of caveolae. Mice lacking this protein do not develop caveolae, among other abnormalities (116). Its function will be discussed in detail below. Caveolin-2 is a 20 kDa protein that commonly co-localises with caveolin-1 and is dependent on the former for adequate membrane insertion and function. It was initially thought to function purely in regulating the transport of caveolin-1, however caveolin-2 knockout mice have been shown to develop pulmonary and skeletal muscle abnormalities without disruption of caveolae (117, 118). It has been associated with the basal-like subtype and poor prognosis breast cancer in two studies (119, 120). Caveolin-3 is a 17 kDa isoform expressed mainly in muscle cells, where its function appears to be analogous to that of caveolin-1 in non-muscle cells (121). Mouse caveolin-3 knockdown models lack muscle cell caveolae and develop signs of muscular dystrophy (122), as well as insulin resistance (123). Caveolin-3 abnormalities have also been associated with several neuromuscular disorders in humans (124).

The functions of caveolae are myriad, and are the subject of ongoing investigation. Caveolae are a site of clathrin-independent endocytosis (125, 126); and are involved in transcytosis in endothelial cells (127, 128). Caveolae, with their high cholesterol content, play a role in intracellular cholesterol homeostasis: caveolin 1 selectively binds to cholesterol (129), while caveolin-negative mutant cells retain excessive intracellular cholesterol (130). Similarly, caveolae may regulate cellular water homeostasis as they express high levels of aquaporins (131). Caveolae and caveolin have also been show to regulate cellular responses to mechanical stimuli and changes in membrane tension (132, 133).
One of the primary putative roles for lipid rafts, and in particular caveolae, is to serve as a platform for numerous signalling pathways. This is facilitated by the large number of signalling proteins that selectively compartmentalize in caveolae. The caveolin scaffolding domain (CSD) of caveolin 1 interacts with several tyrosine kinases (including Src family kinases, MAP kinases, protein kinase A and C), as well as several G protein-coupled receptors, adenylyl cyclase, endothelial nitric oxide synthase (eNOS), and the sodium potassium ATPase (Na⁺ K⁺ ATPase) (134). The “caveolin signalling hypothesis” proposes that caveolae serve to regulate signalling cascades by entrapping both receptors and their downstream effectors (135), while the effect of this altered spatial arrangement may either inhibit (as in c-Src (136)) or promote signalling events (eg G protein signalling). Palmitoylation, or reversible addition of palmitate residues, has been shown to target several proteins to lipid rafts and caveolae. For example, unpublished work from our laboratory has demonstrated that palmitoylation of the hyaluronic acid receptor CD44 functions as a molecular switch regulating the shuttling of CD44 in and out of lipid rafts, and thus its effects on breast epithelial cell migration (Babina et al, personal communication).

The role of caveolae and caveolin in cancer is both controversial and complicated. Caveolin-1 has been described as both a tumour suppressor and promoter, and most likely plays a dual role. Caveolin-1 expression and caveolar density are decreased in both cell lines and tumour samples derived from a range of malignancies, including breast cancer, while caveolin re-expression or
overexpression reduces malignant cell growth in vitro (137-143). A Japanese group has identified a specific caveolin-1 mutation, P132L, that is present in up to 16% of breast cancers and disrupts the caveolin scaffolding domain (144). Caveolin-1 null mice do not develop spontaneous tumours, although they are more susceptible to carcinogen-induced epidermal tumourigenesis (145); while loss of caveolin-1 gene expression in transgenic mammary tumour-prone mice accelerates tumour development (146). This has led to the suggestion that caveolin-1 functions as a transformation suppressor gene (147).

However, certain tumours, such as prostate, bladder, papillary thyroid and oesophageal carcinomas consistently show upregulation of caveolin-1, with expression levels often correlating with markers of aggressiveness (147, 148). Caveolin-1 has also been shown to be overexpressed in multi drug resistant cancer cell lines (149, 150). Caveolin-1 overexpression has additionally been shown to correlate with the metastatic phenotype in several malignancies, including breast cancer (151, 152). It is therefore hypothesized that, in the early stages, caveolin-1 is protective against malignant transformation. However, with tumour progression, re-expression of caveolin may promote tumour cell survival, preventing apoptosis, allowing the acquisition of multi drug resistance, and thus facilitating metastatic spread (153).

1.7.5. The Sodium Potassium ATPase and Cardiac Glycosides

Sodium potassium adenosine triphosphatase (Na⁺ K⁺ ATPase) is a ubiquitous protein that was first discovered by Skou in 1957 (154). It is a transmembrane
protein consisting of three subunits; alpha, beta and a member of the FXYD family. It primarily functions as an ion channel, exporting three sodium ions and importing two of potassium against their concentration gradients in an ATP-dependent reaction, maintaining transmembrane ion concentrations. (155, 156)

The alpha-subunit is a 112-kDa protein and has 4 known subtypes (α1-4). Of these, the α1 isoform is ubiquitously expressed; α2 is expressed primarily by muscle, brain, lung and adipocytes; α3 occurs in cardiac, neural, ovarian and white blood cells; and α4 is expressed solely in spermatozoa. The α subunit is the catalytic unit, with binding domains for ATP and cations. The β subunit, a glycoprotein of 35-kDa, has 3 subtypes (β1-3). It regulates maturation and insertion of the protein into the cell membrane, and also influences ATPase activity. The FXYD family consists of 7 proteins which serve to increase or decrease the ATPase activity; but, unlike the α and β subunits, these are not directly required for pump function (157, 158).
Figure 1.2. The Na\(^+\) K\(^+\) ATPase

Schematic diagram of the Na\(^+\) K\(^+\) ATPase pump illustrating its position spanning the phospholipid membrane. It consists of three subunits; \(\alpha\), \(\beta\) and a member of the FXYD family; and extrudes three Na\(^+\) ions in exchange for two K\(^+\) ions, in an ATP-dependent reaction.

Briefly, the ATPase binds adenosine triphosphate (ATP) and 3 intracellular Na\(^+\) ions. The ATP is hydrolysed, allowing the freed phosphate to phosphorylate the ATPase, inducing a conformational change which transports the Na\(^+\) ions outside the cell. The ATPase then binds 2 K\(^+\) ions, is dephosphorylated, reverting to its initial configuration and transporting the K\(^+\) ions inside the cell. By maintaining the transmembrane ion concentration, Na\(^+\) K\(^+\) ATPase serves to maintain transmembrane potential, driving multiple transport mechanisms and controlling cell volume and osmolality (159). More recently, a secondary role for the Na\(^+\) K\(^+\) ATPase in cell signalling has been recognised, largely based around its interaction with a class of drugs known as cardiac glycosides (160).
Cardiac glycosides are a class of naturally-occurring steroid-like compounds that are produced by a variety of plants, as well as amphibians, and have been used for centuries in medicine. Common examples include digoxin and digitoxin, both from the foxglove plant *Digitalis*; ouabain, from the *Acokanthera* genus of flowering plants; oleandrin, from the *Nerium oleander* shrub; and marinobufagenin, from *Bufo* toads. They function by binding to the α subunit of the Na⁺ K⁺ ATPase and blocking its pumping function. This leads to a build-up of intracellular Na⁺, which in turn delays the extrusion of intracellular Ca²⁺. The increase in intracellular Ca²⁺ increases the length of the cardiac action potential, slowing heart rate; and in parallel elevates sarcoplasmic reticulum Ca²⁺ which increases myocardial contractility (156). Their most common usage is in the treatment of cardiac failure and arrhythmias, with digoxin and digitoxin being the most widely used.

Since the late 1990s, several authors have noted that cardiac glycosides such as ouabain induced changes in cell biology at concentrations that were insufficient to affect Na⁺ K⁺ ATPase pump function (161-163). Since then further work, largely by a group based in the University of Toledo, has established that the Na⁺ K⁺ ATPase functions as a signal transducer, and that binding of cardiac glycosides induces several signalling cascades in a tissue dependent manner. Xie and colleagues have described a “Na⁺ K⁺ ATPase-Src-caveolin signalling complex” (164), whereby Na⁺ K⁺ ATPase interacts with the N terminal of caveolin in caveolae and forms complexes with the nonreceptor tyrosine kinase Src and epidermal growth factor receptor (EGFR). Binding of cardiac glycosides
to Na$^+$ K$^+$ ATPase induces the phosphorylation and activation of Src, which in turn activates EGFR. This has multiple downstream effects, including activation of the Ras/Raf/ERK cascade; recruitment and activation of PI3-kinase; and increased mitochondrial production of reactive oxygen species (165, 166). In fact, Liang et al have identified a non-pumping pool of Na$^+$ K$^+$ ATPase, residing in caveolae and consisting of over half the membrane Na$^+$ K$^+$ ATPase (167). The end results of these signalling cascades include increased contractility and hypertrophy in cardiac myocytes; and apoptosis and reduced proliferation in malignant cells.

In addition to their effects on the Na$^+$ K$^+$ ATPase, cardiac glycosides may also act as oestrogen receptor antagonists. Cardiac glycosides such as ouabain share many structural similarities with 17β-oestradiol (E2) and ethiny1 oestradiol (EE), both of which can also bind to Na$^+$ K$^+$ ATPase (168, 169). Cardiac glycosides block the interaction between oestrogen receptors and 17β-oestradiol. For this reason, these agents may be particularly useful in oestrogen-dependent cancers such as breast cancer (170).

A substance indistinguishable from ouabain has been discovered in human plasma (171), and has since been shown to be synthesised in the adrenal gland (172). This, coupled with the high affinity cardiac glycoside binding site on Na$^+$ K$^+$ ATPase, which is highly conserved across the species, has led to the theory that endogenous ouabain may serve as a hormone regulating cardiac function and blood pressure (159, 173).
1.8. Cardiac Glycosides and Cancer: Clinical Evidence

Cardiac glycosides have been used in traditional medicine for the treatment of several ailments, including malignancies. However, despite the large amount of \textit{in vitro} evidence for their anti-cancer effects, there have been few clinical studies examining these effects. Stenkvist et al redirected attention to the potential anti-breast cancer effects of cardiac glycosides in a 1979 epidemiological study that demonstrated that, of 142 patients with breast cancer, the 28 patients taking cardiac glycosides had tumours that were histologically better-differentiated, and exhibited less distal metastasis at two years (174). Follow-up at 5 years showed a ten-fold reduction in recurrence among those taking cardiac glycosides (175), while 22 year follow-up of 175 patients found that those taking cardiac glycosides had statistically lower disease-related mortality (6% vs 34%) (176).

In marked contrast, Haux et al reviewed 9271 patients taking digitalis, and reported a higher than expected incidence of cancers including breast cancer. However, further analysis revealed that these patients had a pre-existing elevated cancer risk; and that higher serum digitoxin levels correlated with a reduced risk of haematological and urinary tract malignancies (177). Nonetheless, a number of retrospective studies have suggested an increased risk of breast cancer among digitalis users (178-184), mainly ascribed to their oestrogen-mimetic properties. Others have shown no significant association (181, 185). A Medline search yielded eight clinical studies examining the incidence of breast cancer in patients with a history of exposure to cardiac glycosides, outlined in Table 1.4 below. Of these, five were suitable for meta-analysis (1 retrospective cohort study, 4
retrospective case-control studies), with a total number of 303,852 patients. Patients with any history of digitalis exposure had an overall slightly but significantly increased rate of breast cancer compared to patients with no exposure (OR 1.1, 95% CI 1.05-1.16, p <0.001).

This disparity between potential anti-cancer and pro-malignant effects of cardiac glycosides is difficult to explain, but may be related to the selective oestrogen receptor modulating effects of cardiac glycosides previously observed. Of note, only one of the papers we analysed specifically examined breast cancer hormone receptor status. In this paper by Biggar et al, the increased risk of breast cancer in digoxin users was slightly higher for ER positive (RR 1.35) and ER unknown (RR 1.51) cancers compared to ER negative (RR 1.20). Perhaps development of selective Na⁺ K⁺ ATPase ligands with less oestrogenic effects may yield safer anti-cancer agents with more acceptable side effect profiles.
Table 1.4. Studies examining incidence of breast cancer in cardiac glycoside users

<table>
<thead>
<tr>
<th>Study</th>
<th>Population/Design</th>
<th>Findings</th>
<th>Other Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biggar, 2011 Denmark Retrospective cohort (180)</td>
<td>104,648 women using digoxin, 2,144 developed breast Ca (1995-08)</td>
<td>Current users RR 1.39, former users RR 0.91. ER pos 1.35, ER unknown 1.51, ER neg 1.2</td>
<td>137,493 on other cardiac meds-2,658 breast Ca</td>
</tr>
<tr>
<td>Ahern, 2008 Denmark Case control (.78)</td>
<td>5,565 postmenopausal women diagnosed with breast Ca 1991-07; 55650 matched controls</td>
<td>Digoxin OR breast Ca 1.30 (1.39 for 7-18 yrs use)</td>
<td></td>
</tr>
<tr>
<td>Haux, 2001 Norway Case control (177)</td>
<td>SIR breast Ca 1.25 in Digitoxin users, prostate 1.25, leuk/lymph 1.41, all 1.27</td>
<td>Subsequent digitoxin users OR: breast 1.19, all sites 1.21</td>
<td>Higher serum digitoxin correlates with lower incidence leukaemia/lymphoma &amp; urinary tract Ca</td>
</tr>
<tr>
<td>Ewertz, 2001 Sweden Case control (182)</td>
<td>156 cases male breast Ca</td>
<td>468 matched controls</td>
<td>Digoxin OR 2.0</td>
</tr>
<tr>
<td>Lefanfant-Pejoovic, 1990 France/Swiss Case control (183)</td>
<td>91 Male breast Ca, 255 cancer controls</td>
<td>Digitalis use increased incidence (OR 3.8)</td>
<td></td>
</tr>
<tr>
<td>Casagrande et al, 1988 USA Case control (181)</td>
<td>73 male breast Ca vs 73 controls</td>
<td>4 cases vs 10 controls had previous digoxin use (NS)</td>
<td></td>
</tr>
<tr>
<td>Aromaa, 1976 Finland Retrospective Case control (179)</td>
<td>109 case-control pairs on anti-hypertensives</td>
<td>Increased RR with any diuretic use and HF as well</td>
<td>Digitalis RR 1.33-2.67 (higher when prev cancers and differing duration of tx)</td>
</tr>
<tr>
<td>Danielson, 1982 USA Retrospective case control (185)</td>
<td>2 year follow up of 302 women newly diagnosed with breast Ca</td>
<td>RR 1.3 in digitalis users (NS)</td>
<td>No significant association with digitalis</td>
</tr>
<tr>
<td>Study or Subgroup</td>
<td>Digitalis Users</td>
<td>Control</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>------------------</td>
<td>----------------</td>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td></td>
<td>Events</td>
<td>Total</td>
<td>Events</td>
</tr>
<tr>
<td>Casagrande, 1988</td>
<td>4</td>
<td>14</td>
<td>69</td>
</tr>
<tr>
<td>Lenfant-Pejovic, 1990</td>
<td>10</td>
<td>18</td>
<td>81</td>
</tr>
<tr>
<td>Ewertz, 2001</td>
<td>20</td>
<td>52</td>
<td>136</td>
</tr>
<tr>
<td>Aberne, 2008</td>
<td>324</td>
<td>2870</td>
<td>5241</td>
</tr>
<tr>
<td>Biggar, 2011</td>
<td>2144</td>
<td>104648</td>
<td>2658</td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td><strong>107602</strong></td>
<td><strong>196870</strong></td>
<td><strong>100.0%</strong></td>
</tr>
</tbody>
</table>

Total events: 2502
Heterogeneity: $\chi^2 = 21.85, df = 4 (P = 0.0002); I^2 = 82\%$
Test for overall effect: $Z = 3.75 (P = 0.0002)$

**Fig. 1.3. Forest plot of pooled data examining relative risk of breast cancer in digitalis users.**

Five studies examining the incidence of breast cancer after digitalis exposure were analysed using Revman V 5 software were (1 retrospective cohort study, 4 retrospective case-control studies), with a total number of 303,852 patients. Patients with any history of digitalis exposure had an overall slightly but significantly increased rate of breast cancer compared to patients with no exposure (OR 1.1, 95% CI 1.05-1.16, $p < 0.001$).

A number of phase I and II trials are currently underway examining the effect of oleandrin and digoxin products in refractory solid tumours with variable preliminary results (186-189). The results of larger scale studies are eagerly awaited.
1.9. Conclusion and Aims

It is clear from the information presented above that the role of cardiac glycosides in breast cancer is complex, both in vitro and in vivo. While cardiac glycosides have exhibited some promising effects in vitro, their effects in clinical studies are more varied. The reasons for these seeming contractions, and potential mechanisms for harnessing their anti-tumoural effects clinically, must be the subject of future research.

This thesis aims to mechanistically examine the impact of cardiac glycosides on breast cancer models in vitro, with particular attention to the role of lipid rafts and caveolae in modulating these effects. Given the reported localisation of the non-pumping, signalling pool of Na⁺ K⁺ ATPase in caveolae, and the complex role that caveolae and caveolin-1 plays in cancer biology, we reasoned that caveolin-1 might modulate the effects of cardiac glycosides at the Na⁺ K⁺ ATPase. Furthermore, as previous work from our group identified shuttling of CD44 in and out of lipid rafts in migrating versus non-migrating breast cells, we hypothesized that similar shuttling of Na⁺ K⁺ ATPase in and out of caveolae might occur, in response to binding of cardiac glycosides, allowing Na⁺ K⁺ ATPase to interact with downstream signalling effectors.

With this in mind, the following chapters will interrogate this hypothesis, with the following broad aims:
1. To investigate potential anti-neoplastic effects of cardiac glycosides in breast cancer cell lines and primary breast cancer-derived cell cultures; with specific attention to their effects on tumour cell proliferation, migration and cell cycle progression.

2. To determine how potential anti-neoplastic effects are modulated by caveolar lipid rafts; in terms of the physical presence of Na\(^+\) K\(^+\) ATPase in lipid rafts and the influence of manipulation of lipid raft components on the anti-tumoural effects of cardiac glycosides.
Chapter 2

Materials & Methods
2.1. Cell culture

2.1.1. Breast cell lines

The breast cancer cell lines MCF 7 (ER-positive, PR-positive, HER 2-negative, weakly invasive), MDA-MB 231 (ER-negative, PR-negative, HER 2-negative) were used, in addition to primary breast cell cultures generated from breast cancer patients in Beaumont Hospital following ethical approval from Beaumont Hospital Medical Ethics (Research) Committee and informed patient consent. MCF 7 cells were acquired from the European Collection of Cell Cultures (ECACC). MDA-MB 231 cells were acquired from the American Type Culture Collection (ATCC). Laboratory cell line stocks were confirmed to be mycoplasma-free by quarterly testing. The identities of the cell line stocks were periodically verified by Short Tandem Repeat (STR) genetic analysis using Identifiler® PCR kits (BioScience UK). STR profiles that matched each cell line were verified using the ATCC STR profile database (http://www.lgcstandardsatcc.org/ATCCCulturesandProducts/CellBiology/STRPprofileDatabase/tabid/986/Default.aspx).

2.1.2. Cell Culture

All cell culture and manipulations were carried out in a Class II laminar flow hood. MCF 7 cells were cultured in modified Eagle medium (MEM, Sigma) supplemented with 2 mM glutamine, 100 u/mL penicillin, 0.1 mg/mL streptomycin, 1% non essential amino acid solution and 10% fetal bovine serum. MDA-MB 231 cells were cultured in Dulbecco’s modified Eagle medium
(DMEM, Sigma) containing 2 mM glutamine, 100 u/mL penicillin, 0.1 mg/mL streptomycin and 10% fetal bovine serum.

2.1.3. Generation of Primary Cell Cultures

Primary cell cultures were generated as described in (190). Briefly, samples of tumour and normal breast tissues from excised tumour resection specimens were placed in a mixture of 0.5 mg/mL penicillin, 0.5 mg/mL streptomycin and 0.1 mg/mL neomycin (Gibco) in DMEM/ Ham’s F12 for 10 minutes. Samples were diced in a digestion medium (see Appendix) and digested for 2 hours on a mechanical shaker at 37°C. After being allowed to settle in tubes for 5 minutes, the supernatant was aspirated and centrifuged at 1000 rpm for 3 minutes. The pellet was resuspended in digestion medium and spun at 1000 rpm for 3 minutes. This was resuspended in Mammary Epithelial Cell Growth Medium (MEGM, Lonza, see Appendix) and plated onto a 25 cm² tissue culture flask.

2.1.4. Sub Culturing of Cells

Media was removed from flasks and cells were washed with sterile PBS. 2ml of 0.05% trypsin-EDTA was added to the flasks and incubated at 37°C until detachment was visible by light microscopy. When fully detached, trypsin was neutralized with an equal volume of either serum-containing media (for cell lines) or 1x soybean trypsin inhibitor (for primary cell cultures), and the suspension transferred to a sterile 15 ml tube. This was centrifuged at 1000 rpm for 3 minutes. The supernatant was aspirated and the pellet resuspended in media. Cells were counted using a haemocytometer under light microscopy at
10x magnification and the number of cells per mL estimated. Cells were then seeded at the indicated densities into tissue culture flasks or plates.

2.2. Functional Assays

2.2.1. MTT Proliferation Assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) is a tetrazolium dye that is reduced by viable cells to form purple formazan crystals. The resulting purple colouration can be used to estimate the number of viable cells.

Cells were seeded in 96 well plates at 2,000 per well for MDA-MB 231 or 5,000 per well for MCF 7 and allowed to attach overnight in 200 µL media. The following day the medium was removed and replaced with fresh medium containing digoxin, ouabain or oleandrin in a range of concentrations. DMSO at a volume equivalent to that in the highest concentration of drug was used as vehicle control. 5 hours before each scheduled timepoint, 20 µL MTT reagent (5 mg/mL in PBS) was added to each well at a final concentration of 0.5 mg/mL. At each time point (24, 48, 72 hours), the media was removed and replaced with 200 µL DMSO and mixed to solubilise formazan crystals. The plate was returned to the incubator for 5 mins before reading in a Perkin Elmer plate reader at 450nm. Results were graphically plotted as optical density values normalised to averaged controls.
2.2.2. Migration Assay

Cells were grown in 24 well plates and allowed to adhere overnight. The next day, medium was aspirated and replaced with complete media containing digoxin, ouabain, oleanadrin or vehicle control for 72 hours. After 72 hours media was aspirated and replaced with PBS. Monolayers were scratched once with a sterile P200 pipette tip attached to suction before the PBS was aspirated and replaced with the original media and treatment. The wounds were photographed at hourly intervals up to 6 hours under light microscopy at 20x and percentage wound closure compared to time zero was calculated using Image J version 10.2 software.

2.2.3. Silencing of Caveolin-1 and Flotillin-1 by siRNA

Small interfering RNA (siRNA) against caveolin-1 (ThermoScientific), flotillin-1 (Santa Cruz) or a universal negative control (Sigma) were transfected into breast cells following the manufacturer’s protocol. In brief, cells were seeded in 96 well plates at 5,000 (MDA-MB 231) or 10,000 (MCF 7) per well in 100 μL antibiotic-free serum-positive medium and allowed to adhere for 12 to 16 hours. A mixture of DharmaFECT-1 transfection reagent (Dharmacon) and siRNA was made up as follows: for each well, a volume of 0.5 μL of 5 μM target or nonsense siRNA in 9.5 μL serum free media was mixed with either 0.1 (MDA-
MB 231) or 0.2 μL (MCF 7) transfection reagent in 9.99 or 9.98 μL serum free media (ie 20 μL per well) and allowed to stand for 20 minutes. Blank solution containing no siRNA was also made up. Culture media was aspirated from each well and replaced with 20 μL of the appropriate transfection mixture; as well as 80 μL per well antibiotic-free medium containing digoxin, ouabain, oleandrin or vehicle control (end concentration 25 nM siRNA, 0.1/0.2 μL transfection reagent per well). Cell proliferation was determined by MTT assay as above, with the quantity of MTT solution halved to 10 μL in view of the reduced amount of medium used per well, to achieve the desired final concentration of 0.5mg/mL.

2.3. Protein Analysis

2.3.1. Preparation of Whole Cell Lysates

Confluent cells were washed once in PBS, then subsequently twice in ice cold PBS, each wash lasting ten minutes. Ice-cold Relax buffer (see appendix), containing 1% each of Triton-X100, protease inhibitor cocktail, phosphatase inhibitor cocktail 2 & 3 (Sigma) was added. Cells were scraped and dounced 20 times in a tight-fitting Dounce homogeniser and centrifuged at 1500xg, 4°C for five minutes. Supernatants were snap frozen in liquid nitrogen and stored at minus 80°C prior to use.

2.3.2. Protein Quantification

Protein concentration was assayed using bicinchoninic acid (BCA) protein assay kits (Thermo Scientific). 10 μL of each lysate of interest was added to wells of a
96 well plate, as well as serial dilutions of 5mg/mL bovine serum albumin stock to obtain a standard protein concentration curve. BCA reagents A and B were mixed in a 50:1 ratio prior to use, and 100 μL was added to each well of interest. This was covered and incubated at 37°C until adequate colour change had occurred (approx 30 minutes), before reading at 550 nM on a Perkin Elmer plate reader. Protein concentration of each sample was calculated from the standard curve.

2.3.3. SDS-PAGE and Western Blot Analysis

SDS gels were made at the appropriate concentrations (see appendix). Lysates were mixed with 4X Laemelli sample buffer (see appendix) and heated to either 60 or 100°C for 5 minutes to denature the protein. As per manufacturer’s instructions, the mouse anti-Na⁺K⁺ ATPase antibody used was incompatible with heating to 100°C; consequently heating to 60°C was used when this was the protein of interest. Equal quantities of protein were loaded in each gel lane. Gels were run at 40 milliamps constant current per gel and transferred to 0.2 μm pore nitrocellulose membranes in cold transfer buffer (see appendix) at a constant 100 volts for one hour.

Protein transfer was confirmed using Ponceau S (see appendix) staining. The membranes were blocked in blocking buffer (Tris buffered saline [see appendix] containing 1% Tween-20 [TBS-T] and either 5% nonfat dry milk or 5% bovine serum albumin [for phosphorylated proteins]) for one hour, then incubated overnight in blocking buffer containing primary antibody at 4°C. Membranes were then washed three times for 10 minutes in TBS-T before being incubated in
horseradish peroxidase-conjugated secondary antibody in blocking buffer for one hour at room temperature. The membranes were then washed 6 times for five minutes each in alternatating solutions of TBS and TBS-T. Bound antibody was detected by peroxidase-catalysed enhanced chemiluminescence on photographic paper and developed in the dark. Images were analysed by densitometry using ImageJ version 10.2 software.

2.3.4. Stripping of Antibodies from Nitrocellulose Membranes
Membranes were washed six times for five minutes each in TBS-T. In the fume hood, 10 mL stripping buffer (see appendix) was added to each membrane and incubated in a sealed box at 60° C. Stripping buffer was then discarded and membranes were washed a further six time for five minutes each in TBS-T. Membranes were subsequently blocked and probed as per Section 2.3.3.

2.3.5. Immunofluorescence confocal microscopy
Cells were seeded at approximately 50,000 per well on sterile glass coverslips in wells of 24 well culture plates. At 50% confluence media was aspirated and replaced with media containing digoxin, ouabain, oleandr in or vehicle control for 72 hours. Coverslips were removed, washed three times in PBS and fixed in 100% methanol at -20°C for 20 minutes. Coverslips were washed three times with PBS and blocked for one hour at room temperature in blocking buffer (5% goat serum in PBS). Coverslips were washed three times with PBS and incubated for one hour in blocking buffer containing primary antibody (see appendix). Coverslips were washed three times with PBS and incubated in blocking buffer
containing 1:1000 secondary antibody conjugated to fluorophore for one hour (Alexa-Fluor, see appendix). Coverslips were washed three times with PBS and incubated in PBS containing 1 mg/mL 4′, 6-diamidino-2-phenyl-indole (DAPI) for ten minutes. Coverslips were washed three times with PBS, allowed to dry, mounted onto glass slides with p-phenylenediamine/PBS/glycerol (0.01:1:1 v/v/v/v) and sealed. These were then examined and photographed using a Zeiss LSM-710 meta confocal microscope. Detector gain settings were identical for cross-comparison across control/treated conditions. Analysis of the percentage of Na⁺ K⁺ ATPase co-localising with caveolin-1 was performed using Image J software with co-localisation and area quantification plugins downloaded from (191).

2.4. Cell cycle analysis

2.4.1. Flow cytometry

Confluent monolayers of cells in 10cm dishes pretreated with digoxin, ouabain, oleandrin or vehicle control for 72 hours were washed with PBS and trypsinnized as described in Section 2.1.4. Detached cells were suspended in 7 mL PBS and centrifuged at 200xg for 3 minutes. Pellets were re-suspended in 1.5 ml PBS containing 0.2% EDTA and passed through 50 μM CellTrics cell filters (Partec). These were spun at 200xg for 2 mins, and the supernatant aspirated. Pellets were resuspended in 1 mL ice cold 100% ethanol and fixed overnight at -20°C. Solutions were subsequently centrifuged at 200xg for 5 mins, supernatant aspirated and pellets resuspended in PBS. This was spun again at 200xg for 2
mins and supernatants aspirated. This wash was repeated again, and pellets were resuspended in 500μL 0.2% EDTA containing 50 μL RNAse and incubated at 37°C for 30 minutes. 25 μL of propidium iodide (50 μg/mL in dH₂O) was added, and samples stored at 4°C. Samples were filtered again and analysed in a Becton Dickinson FACScalibur flow cytometer to estimate the percentage of cells at each stage of cell cycle. No gating was used, and the instrument settings are outlined in Table 2.1 below.

Table 2.1. Flow cytometer instrument settings used for MCF 7 and MDA-MB 231 cells

<table>
<thead>
<tr>
<th>MCF 7</th>
<th>Voltage</th>
<th>MDA-MB 231</th>
<th>Voltage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detector</td>
<td></td>
<td>Detector</td>
<td></td>
</tr>
<tr>
<td>FSC</td>
<td>E00</td>
<td>FSC</td>
<td>E00</td>
</tr>
<tr>
<td>SSC</td>
<td>350</td>
<td>SSC</td>
<td>350</td>
</tr>
<tr>
<td>FL 1</td>
<td>600</td>
<td>FL 1</td>
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<tr>
<td>FL 2</td>
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<td>FL 2</td>
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</tr>
<tr>
<td>FL 3</td>
<td>650</td>
<td>FL 3</td>
<td>650</td>
</tr>
</tbody>
</table>

2.4.2. RNA Extraction from MCF 7 Cells

RNA was extracted using the RNeasy® extraction kit (Quiagen), according to the manufacturer’s instructions. Briefly, MCF 7 cells were seeded in wells of a 6 cm plate at 300,000 per well and treated with digoxin or ouabain for 72 hours. Monolayers were scraped in 350μL RLT lysis buffer and triturated using a 26
gauge needle and syringe. This was mixed with 1 volume of 70% ethanol and centrifuged in RNeasy spin columns for 15 seconds at 8000xg, discarding the flow-through. 700 μL of buffer RW1 was added to the columns and centrifuged for 15 seconds at 8000xg, discarding the flow-through. 500 μL of buffer RPE was added to the columns and centrifuged for 15 seconds at 8000xg, discarding the flow-through. A further 500 μL of buffer RPE was added to the columns and centrifuged for two minutes at 8000xg, discarding the flow through. The columns were placed in new Eppendorf tubes. 40 μL of RNase-free water added and centrifuged for 1 minute at 8000xg to elute RNA. This was repeated with a further 40 μL RNase-free water. RNA concentration was verified using a Nanodrop spectrophotometer (Thermo Scientific), with an A260:280 ratio ≥ 2.0 defining adequate purity.

2.4.3. Reverse Transcription

RNA was converted to cDNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems) according to the manufacturer’s instructions. Briefly, 2 μg sample RNA was mixed with 10 μL 2X RT buffer, 1 μL 20X enzyme mix and nuclease-free water to a total volume of 20 μL and briefly centrifuged at low speed. This was incubated in a PTC-200 Peltier thermal cycler at 37°C for 60 mins and heated to 95°C for 5 mins to arrest the reaction. Complementary DNA (cDNA) concentration and purity was verified using a Nanodrop spectrophotometer (Thermo Scientific), with an A260:280 ratio ≥ 1.8 defining adequate purity.
2.4.4. Real Time Polymerase Chain Reaction

TaqMan® Fast Real time polymerase chain reaction (rtPCR) plates were custom-prepared with primers for a variety of positive and negative regulators of the cell cycle (see Table 2.2.) and loaded according to the manufacturer’s instructions. Each well was loaded with 5 μL of nuclease free water containing equal quantities of cDNA; and 5 μL Taqman® gene expression master mix. The plate was covered with optical adhesive film and centrifuged at 1000 RPM for 1 minute. The plate was transferred to an Applied Biosystems 7500 Fast rtPCR System thermal cycler. Plates were heated to 95°C for 10 minutes before undergoing 40 cycles of heating to 95°C for 15 seconds and cooling to 60°C for 1 minute. The resulting number of cycles to amplify each gene of interest was calculated relative to 18S ribosomal RNA (rRNA) as control gene; this constituted delta CT (ΔCT). The ΔCT of each gene in the treated samples were further normalised to that of their equivalent gene in the untreated samples to give a value for delta delta CT (ΔΔCT) to determine relative gene expression between controls and treatments.
Table 2.2. Cell cycle regulatory genes, proteins and primers used in rtPCR

<table>
<thead>
<tr>
<th>GENE</th>
<th>PRIMER CODE (INVITROGEN)</th>
<th>PROTEIN</th>
<th>FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>18s</td>
<td>Hs999999901_s1</td>
<td>N/A</td>
<td>Ribosomal RNA: control gene</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>Hs00355782_m1</td>
<td>p21</td>
<td>Cell cycle inhibitor</td>
</tr>
<tr>
<td>CDKN1B</td>
<td>Hs00153277_m1</td>
<td>p27</td>
<td>G1 phase regulator</td>
</tr>
<tr>
<td>TP53</td>
<td>Hs01039246_m1</td>
<td>p53</td>
<td>Tumour suppressor</td>
</tr>
<tr>
<td>PCNA</td>
<td>Hs00696862_m1</td>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
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<td>PTEN</td>
<td>Hs02621230_s1</td>
<td>PTEN</td>
<td>Tumour suppressor</td>
</tr>
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<td>CDK4</td>
<td>Hs00262861_m1</td>
<td>CDK4</td>
<td>G1 Regulator</td>
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<tr>
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<td>CDK6</td>
<td>G1 Regulator</td>
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<td>CCNB1</td>
<td>Hs00259126_m1</td>
<td>Cyclin B1</td>
<td>M Regulator</td>
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2.5. Lipid Raft Analysis

Lipid raft domains can be extracted from cell lysates by isopycnic sucrose density gradient fractionation. Two methods for isolating raft fractions have been described, namely using either Triton X100 detergent or high pH sodium carbonate (detergent-free) to solubilise non-raft proteins. Each method has its limitations, in that each method alters the retrieval of some proteins. As the Triton X100 method has been previously reported to solubilise Na\(^+\) K\(^+\) ATPase (192), and as our preliminary work with this method resulted in insufficient Na\(^+\) K\(^+\) ATPase retrieval, we abandoned this in favour of detergent-free raft extraction, described below.

2.5.2. Detergent-Free Lipid Raft Isolation

Confluent monolayers of MCF 7 and MDA-MB 231 cells in 10cm dishes, treated with either digoxin or ouabain (40 nM for 72 hours), were washed three times x 10 minutes in ice cold PBS before scraping in 500 mM Na\(_2\)CO\(_3\) containing 1% protease inhibitor cocktail (Sigma). Cells were disrupted using 20 strokes of a tight-fitting Dounce homogeniser and triturated 20 times with a 22-gauge needle and syringe. Lysates were rotated end-over-end for 25 minutes at 4°C to allow micelles to form.

1 L Hanks Buffered Salt Solution was prepared freshly and supplemented with
4.17 mM NaHCO₃ and 10 mM HEPES, pH 7.4. This was combined 1:1 with 500 mM Na₂CO₃ and used to prepare 90%, 30%, 20% and 5% (w/v) sucrose solutions. 2.2 ml lysate was mixed with an equal volume of 90% sucrose and pipetted into a microcentrifuge tube. 2.6 ml 30% sucrose was layered on top of this, followed by 2.6 ml each of 20% and 5% sucrose. Ultracentrifuge tubes were transferred to a Beckmann Sw41 ultracentrifuge and spun at 39000 rpm for 19 hours at 4°C. Sucrose gradients were then manually fractionated into 1 ml fractions from the top (low-high sucrose density), and pellets solubilised in 500 μL 0.5% SDS.

Fractions were analysed by the following methods;

- Sucrose densitometry: 2 μL of each fraction was analysed using an Eclipse handheld refractometer and the percent sucrose was determined to confirm linearity of sucrose gradients.

- Alkaline phosphatase activity: 20 μL of each fraction was pipetted into wells of 96 well plates. 200 μL of SigmaFAST p-nitrophenyl Phosphate/ Tris buffer solution (Sigma) was added and incubated at 37°C in darkness. The resultant colour change was analysed in a Perkin Elmer spectrophotometer at 405 nm. Yellow colouration indicated alkaline phosphatase activity, with peak alkaline phosphatase activity predicted to occur in lipid raft fractions.

- Western blot (see Section 2.3. above)

2.5.3. Pharmacological Manipulation of Lipid Raft Integrity
Cells were seeded in 96 well plates at densities of either 2,000 per well (MDA-MB 231) or 5,000 per well (MCF 7) in 200 μL media and allowed to attach overnight. The following day, media was aspirated and replaced by complete media containing the lipid raft-disruptive agent methyl β cyclodextrin (MBCD, 13.2 mM, Sigma), with or without cholesterol (0.4 mg/mL, Sigma); or complete media alone. These treatment concentrations were defined in (193). After two hours this was again aspirated and replaced with complete media containing digoxin, ouabain, oleandrin or vehicle control and incubated for 43 hours. This was then aspirated and replaced with media containing both cardiac glycosides/vehicle control and MBCD with or without cholesterol as per pre-treatment, as well as 20 μL per well MTT, and incubated in the dark for a further 5 hours. Cells were then assayed as per Section 2.2.1. above.

2.6. Statistical Analysis

Statistical analysis was performed using unpaired two-tailed equal or unequal variance Student t-tests as calculated using Microsoft Excel, version 12.2.0 for Mac, on raw data derived from multiple replicates. Unless otherwise stated, all results presented in the following chapters represent pooled results of three independent experiments.
Chapter 3

Effects of Cardiac Glycosides on Functional Cellular Behaviours Relevant to Breast Cancer
3.1. Introduction

Cancer research has long relied on in vivo and in vitro models to study tumour biology at the molecular level. A particular challenge in the study of biological effects of cardiac glycosides is the well-documented insensitivity of rodent Na\(^+\) K\(^+\) ATPase to cardiac glycosides (194). This has precluded meaningful evaluation of cardiac glycosides in murine in vivo cancer models, as well as limiting the range of cell lines available for in vitro work to human-derived lines.

3.1.1. Overview of Cell Lines Used in This Thesis

Although some of the work presented in this thesis was undertaken on primary cell cultures derived from breast cancer patients, the majority was undertaken on commercially available, immortalized cell lines. These offer the advantages of cost-effective, readily available, uniform cell populations that are genetically well-characterized and grow readily and predictably in vitro. Primary breast cancer-derived cell cultures are genetically heterogeneous, difficult, time-consuming and expensive to extract, grow unpredictably, and have a limited useful lifespan in vitro. Indeed, since the isolation of the first breast cancer cell line in 1958, only around 100 permanent cell lines have been established, with a reported success rate as low as 0.7% (195). The majority of these are derived from metastases, of which pleural effusions are the most common sources. Notwithstanding the inherent risk of acquired differences between primary and secondary tumours, pleural effusions offer large numbers of isolated tumour cells, with minimal contaminants such as fibroblasts compared to primary tumour-derived colonies.
A number of methods have been described for generating immortalized cell lines. Tissue samples can be minced and treated with proteinases to digest the extracellular matrix, releasing the cells of interest, which are then grown in culture medium. Alternatively, tissue samples can be directly incubated in media, allowing progenitor cells to grow out, which are then isolated and cultured. This is known as explant culture. In the case of blood and liquid suspensions such as pleural or ascitic fluid, cells can be isolated and cultured directly. Normal cells are subject to a maximum number of divisions, known as the Hayflick limit, before entering senescence, whereby cells remain viable but proliferation ceases (196). This is dictated by the presence of telomeres, nucleotide sequences at the end of each strand of DNA that have been likened to plastic toggles on shoelaces. As eukaryotic cells are unable to replicate the full length of a DNA strand, a number of base pairs are lost with every cell division, leading to progressive shortening and eventual loss of telomeres resulting in senescence or apoptosis. Many malignant cells express telomerase, an enzyme that maintains and lengthens telomeres and is usually expressed in stem cells and germ cells, allowing them to continue to divide indefinitely and to be considered immortal.

The MCF 7 cell line was initially isolated in 1973 by the Michigan Cancer Foundation (197), and was the first mammary carcinoma cell line in widespread usage. It was extracted from a pleural effusion of a 69 year old Caucasian woman with metastatic hormone-receptive invasive ductal carcinoma. It has been subsequently characterized as ER- and PR-positive, and HER2-negative. Its proliferative rate is moderate, and it is only weakly tumourigenic in mice (conditional upon additional oestrogen supplementation) (198). MCF 7 has been
characterized as consistent with the luminal A molecular subtype of breast cancer, and morphologically represents a well-differentiated adenocarcinoma.

The MDA-MB 231 (M.D. Anderson- Metastatic Breast) cell line was isolated from the pleural effusion of a 51-year-old Caucasian female with metastatic breast cancer. It is a poorly differentiated, highly proliferative, highly invasive cell line, and is ER-, PR- and HER2-negative. It expresses several morphological and genetic markers of EMT, and is consistent with the basal molecular subtype of breast cancer (199).

The above two cell lines, in addition to the cell line T-47D, are the subjects of over two thirds of all abstracts reporting work on breast cancer-derived cell lines appearing on Medline (200). Foundation studies using these models have provided invaluable mechanistic insight into signalling pathways that drive cancer initiation and progression. However, the use of immortalized cell lines as breast cancer models is not without its drawbacks. The metastatic origin of most cell lines implies a phenotype inherently different from that of the primary tumour, thus these cell lines may not faithfully represent events prior to the development of metastasis. The process of isolating an immortalized cell line selects only the clonal population that exhibits most proliferation under a given set of artificial conditions. Indeed, the in vitro environment, which usually consists of a clonal population adherent to a flat plastic surface, bathed in nutrient-rich, antibiotic-containing media; is quite different to the natural physiological milieu in which polyclonal tumour cells proliferate in three dimensions, interacting with both normal epithelial cells and a complex
supporting stroma (as described in Chapter 1). Furthermore, given the reliance of a huge number of researchers on a small number of cell lines, cross-contamination between cell lines is an important problem. One study has suggested that as high as 18% of cell lines may be contaminated (201). This necessitates extreme vigilance and rigorous aseptic technique on the part of the researcher, as well as frequent genetic verification of cell lines. In addition, subtle genetic and phenotypic changes that occur after repeated passaging may be difficult to detect. For all these reasons, our studies took the approach of combining cell line data with primary culture data where possible.

3.1.2. Primary Breast Cancer Derived Cell Cultures

The use of primary breast cancer derived cell cultures, as described by Donatello et al. (190), offers some advantages compared to immortalized cell lines. Firstly, they allow researchers to use cells from the primary tumour, or indeed, from specific metastases as desired, and allow comparison of both tumour and adjacent non-tumour cell populations. Epithelial cells are usually not cultured in isolation, but rather in conjunction with supporting cells such as fibroblasts, that allow closer approximation of physiological conditions. Similarly, tumour epithelial cells are less likely to represent a single clonal colony due to the lower number of passages. In addition, primary samples collected at source benefit from detailed individual histopathology reports and clinical data usually missing from commercial cell lines.
Regardless, primary breast cancer-derived cell cultures present a number of specific disadvantages in addition to the limitations inherent in all *in vitro* work. Compared to immortalized cell lines, primary cultures are relatively much slower growing, with a finite lifespan, ceasing proliferation after only a few passages. This limits the quantities of useful cells and protein available for experimentation. Only a minority of samples received ever yield sufficient cellular material for experimentation, and their culture is relatively costly and time consuming. As each successful primary culture is derived from a unique tumour, it is also difficult to assemble several replicates of the less common variants, such as triple negative tumours. Indeed it is impossible to perform truly identical replicate experiments on even the commoner subtypes, as a pathology report cannot fully characterize a tumour. Nevertheless, primary breast cancer-derived cell cultures offer an extremely useful adjunct to experimentation on immortalized cell lines, and are used in a number of figures presented below.
3.2. Specific Aims of This Chapter

Broadly speaking, this chapter aims to document the functional effects of cardiac glycoside treatment on the malignant phenotype, while Chapter Four aims to examine subcellular changes underlying these effects. While there are several known cardiac glycosides, we limited our studies to three representative agents. Digoxin, being by far the most widely used agent in current medical practice, was chosen to allow an element of clinical correlation. Ouabain is the most commonly studied Na$^+$ K$^+$ ATPase antagonist in vitro, and is the subject of much of the available literature. Oleandrin was chosen as its putative anti-cancer effects in non-breast malignancies are currently the subject of clinical trials.

Aim 1 was to determine the effect of cardiac glycoside treatment on cellular proliferation, using both MCF 7 and MDA-MB 231 cell lines, in addition to primary breast cancer-derived cell cultures.

Aim 2 was to examine the effects of cardiac glycoside treatment on breast cancer cell cycle regulation and arrest.

Aim 3 was to examine the effects of cardiac glycosides on breast cancer collective cell migration, using MCF 7 and MDA-MB 231 cells.
3.3 Results

3.3.1. Determination of the Effects of Cardiac Glycoside Treatment on Breast Cancer Cell Proliferation

As one of the most important aspects of the malignant phenotype is disordered cell growth, we began by examining the effects of cardiac glycosides on cell proliferation. Subconfluent MCF 7 and MDA-MB 231 cells were treated with digoxin, ouabain and oleandrin at a range of concentrations and subjected to proliferation assays at 24, 48 and 72 hours after treatment. In brief, subconfluent adherent cells were individually treated with cardiac glycosides (digoxin and ouabain 5-500 nM; oleandrin 50-500 nM) before the addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a yellow compound which is reduced to purple insoluble formazan crystals by reductases in the cytosol of metabolically active cells. The colour change is then read by spectrophotometry, with a higher absorption indicative of higher metabolic activity. The selected concentrations of cardiac glycosides were chosen to range from clinically well-tolerated to toxic.

As shown in Figures 3.2 – 3.4, the weakly invasive, ER- & PR-positive cell line MCF 7 exhibited statistically significant concentration- and time-dependent reductions in proliferation upon treatment with all three cardiac glycosides compared to vehicle controls.
Figure 3.1. Digoxin induces concentration- and time-dependent reductions in MCF 7 cellular proliferation.

MCF7 cells were seeded on 96 well plates and allowed to adhere overnight before treatment with digoxin (5-500nM) relative to vehicle control (0.005% DMSO). MTT proliferation assays were performed at 24, 48 and 72 hours. Reductions in proliferation compared to vehicle control were observed in response to digoxin at 24h (A), 48h (B) or 72h (C). At 24 hours, 150 nM digoxin significantly reduced proliferation compared to 40 nM or less, while 500 nM reduced proliferation compared to 70 nM or less. At 48 hours, doses of 40 nM or more significantly reduced proliferation compared to lower doses. At 72 hours, 70 nM digoxin significantly reduced proliferation compared to 10 nM or lower, while treatment with 150 or 500 nM reduced proliferation compared to 40 nM or lower. (D) confirms a reduction in proliferation in response to 150nM digoxin at 48 and 72 hours compared to 24 hours. Each graph represents the pooled results of three experiments, with error bars representing the standard error of the mean.
(*p < 0.05; **p < 0.01; ***p < 0.001, compared to vehicle control, by two-tailed Student's unpaired t-test).

Figure 3.2. Ouabain induces concentration- and time-dependent reductions in MCF 7 cellular proliferation

MCF 7 cells were seeded on 96 well plates and allowed to adhere overnight before treatment with ouabain (5-500 nM) relative to vehicle control (0.005% DMSO). MTT proliferation assays were performed at 24, 48 and 72 hours. Reductions in proliferation compared to vehicle control were observed in response to ouabain at 24h (A), 48h (B) or 72h (C). At 24 hours, treatment with ouabain 150 nM significantly reduced proliferation compared to 90 nM. At 48 hours, treatment with 40 nM ouabain significantly reduced proliferation compared to 5 nM, 90 nM significantly reduced proliferation compared to 40 nM
or less, 150 nM significantly reduced proliferation compared to 90 nM or less, and 500 nM significantly reduced proliferation compared to 150 nM or less. At 72 hours, treatment with 150 or 500 nM ouabain significantly reduced proliferation compared to treatment with 40 nM or less. (D) confirms a time-dependent reduction in proliferation in response to 150nM ouabain at 48 and 72 compared to 24 hours. Each graph represents the pooled results of three experiments, with error bars representing the standard error of the mean. (*p<0.05; **p <0.01; ***p<0.001, compared to vehicle control, by Student’s two-tailed unpaired t-test).
Figure 3.3. Oleandrin induces concentration- and time-dependent reductions in MCF 7 cellular proliferation.

MCF7 cells were seeded on 96 well plates and allowed to adhere overnight before treatment with oleandrin (50-500nM) relative to vehicle control (0.005% DMSO). MTT proliferation assays were performed at 24, 48 and 72 hours. Reductions in proliferation were observed in response to oleandrin at 24h (A), 48h (B) or 72h (C). At 24 hours, treatment with 250 nM oleandrin significantly reduced proliferation compared to treatment with 50 nM. At 48 hours, treatment with 150 nM oleandrin significantly reduced proliferation compared to treatment with 50 nM, 250 nM significantly reduced proliferation compared to 150 nM or less, and 500 nM significantly reduced proliferation compared to 250 nM or less. At 72 hours, treatment with 150 or 250 nM oleandrin significantly reduced proliferation compared to 50 nM, while treatment with 500 nM significantly reduced proliferation compared to 250 nM. (D) Confirms a time dependent
reduction in proliferation in response to 250nM oleandrin at 48 and 72 hours compared to vehicle control. Each graph represents the pooled results of three experiments, with error bars representing the standard error of the mean. (*p<0.05; **p <0.01; ***p<0.001, compared to vehicle control, by Student’s two-tailed unpaired t-test)

Having established that a weakly-invasive breast cancer cell line exhibited anti-proliferative responses to cardiac glycosides, we next tested the sensitivity of an invasive cell line. In contrast to MCF 7 cells, the triple-negative, highly-invasive MDA-MB 231 cell line exhibited lower sensitivity to cardiac glycosides (Figures 3.4-3.6). Specifically, significant anti-proliferative effects were seen only at high concentrations of digoxin (over 40 nM), with a small increase in proliferation seen with 10 nM relative to 5 nM at all three timepoints. Ouabain treatment induced an increase in proliferation at 10 nM at day 3, with inhibition at concentrations of 40 nM or more, and an almost linear concentration-response curve at day 2. In oleandrin-treated cells, inhibition of proliferation only occurred at doses greater than 50 nM, or 150 nM at day 3. With all three cardiac glycosides there was a somewhat delayed response relative to that in MCF 7s, with a linear concentration-response curve only appearing after 48 hours of treatment. There was a tendency towards time-dependent anti-proliferative effects towards the higher end of the concentration scale (eg digoxin and ouabain 150 nM, oleandrin 250 nM).
Figure 3.4. High dose digoxin induces concentration- and time-dependent reductions in MDA-MB-231 cellular proliferation.

MDA-MB 231 cells were seeded on 96 well plates and allowed to adhere overnight before treatment with digoxin (5-500nM) relative to vehicle control (0.005% DMSO). MTT proliferation assays were performed at 24, 48 and 72 hours. Reductions in proliferation were observed in response to digoxin at 24h (A), 48h (B) or 72h (C). At 24 and 48 hours, treatment with 500 nM digoxin significantly reduced proliferation compared to treatment with 150 nM or below. (D) shows a time-dependent reduction in proliferation in response to 150nM digoxin at 48 and 72 hours compared to vehicle control. Each graph represents the pooled results of three experiments, with error bars representing the standard error of the mean. (*p < 0.05; **p <0.01; ***p<0.001, compared to control, by Student’s two-tailed unpaired t-test).
Figure 3.5. High dose ouabain induces concentration- and time-dependent reductions in MDA-MB-231 cellular proliferation.

MDA-MB 231 cells were seeded on 96 well plates and allowed to adhere overnight before treatment with ouabain (5-500nM) relative to vehicle control (0.005% DMSO). MTT proliferation assays were performed at 24, 48 and 72 hours. Reductions in proliferation were observed in response to ouabain at 24h (A), 48h (B) or 72h (C). At 24 and 48 hours, treatment with 500 nM ouabain significantly reduced proliferation compared to treatment with 90 nM or lower. In addition, at 48 hours treatment with 90 nM ouabain significantly reduced proliferation compared to treatment with 10 nM or less. (D) shows a time-dependent reduction in proliferation in response to 150nM ouabain at 48 and 72 hours compared to vehicle control. Each graph represents the pooled results of three experiments, with error bars representing the standard error of the mean.
(*p< 0.05; **p <0.01; ***p<0.001, compared to control, by Student’s two-tailed unpaired t-test).

**Figure 3.6. High dose oleandrin induces concentration- and time-dependent reductions in MDA-MB-231 cellular proliferation.**

MDA-MB 231 cells were seeded on 96 well plates and allowed to adhere overnight before treatment with oleandrin (50-500nM) relative to vehicle control (0.005% DMSO). MTT proliferation assays were performed at 24, 48 and 72 hours. Reductions in proliferation were observed in response to oleandrin at 24h (A), 48h (B) or 72h (C). At 48 and 72 hours, treatment with 500 nM oleandrin significantly reduced proliferation compared to treatment with 250 nM or less. (D) shows a time-dependent reduction in proliferation in response to 250nM
oleandrin at 48 and 72 hours compared to vehicle control. Each graph represents the pooled results of three experiments, with error bars representing the standard error of the mean. (*p < 0.05; **p < 0.01; ***p < 0.001, compared to control, by Student’s two-tailed unpaired t-test).

3.3.2. Determination of the Effects of Cardiac Glycoside Treatment on Primary Breast Cancer-Derived Cell Cultures

Having observed encouraging anti-proliferative effects in both hormone receptor-positive and -negative cell lines, we sought to translate these results into a more patient relevant setting by determining whether comparable effects could be observed in primary breast cancer derived cultures, a closer approximation of the true tumour microenvironment. Both tumour and adjacent non-tumour epithelial cells were isolated from surgical specimens as described in chapter 2, passaged and seeded in 96 well plates. In contrast to the previously described work on immortalized cell lines, cells were allowed to reach 50% confluence before being treated with cardiac glycosides and subjected to MTT proliferation assays 24, 48 and 72 hours after treatment. This reflects the slower growth rate of primary cell cultures.

Due to the unpredictable nature both of clinical sample availability and of primary cell culture growth in vitro, it was not possible to obtain multiple specimens of all histological subtypes, as detailed in the figures below. In light of this, the results shown below must be interpreted with caution, although
similar trends to the work in immortalized cell lines may be observed. Specifically, as shown in Figure 3.7; ER- and PR- positive, HER2-negative derived cells were exquisitely sensitive to digoxin treatment even at 5 nM, but with a small spike in proliferation occurring at 10 nM. There appeared to be an increase in proliferation at 5 and 10 nM ouabain at day 1, with linear concentration-response effects occurring at higher concentrations and by day 3. Oleandrin inhibited proliferation at concentrations greater than 50 nM at day one, with severe inhibition (>60%) occurring at all concentrations by day 3. In contrast, cultures derived from non-tumour epithelial cells at the margins of ER- and PR-positive, HER2-negative tumours (Figure 3.8) were somewhat more resistant to the effects of digoxin, with a small increase in proliferation seen at 10 nM treatment and with significant anti-proliferative effects only occurring at 100 nM. These cells appeared more susceptible to the effects of low dose ouabain than their associated tumour cells, although effects were similar at or above 40 nM. These cells were more sensitive to oleandrin at 24 hours, but more resistant to the anti-proliferative effects of low dose oleandrin (50 nM) at day 3.

Invasive lobular carcinoma-derived cells (Figure 3.9) showed significant but non-linear anti-proliferative responses to all three cardiac glycosides at all concentrations tested. ER- and PR-negative, HER2-negative carcinoma-derived cells (Figure 3.10) showed significant, but non-linear, reductions in proliferation in response to all three cardiac glycosides at day 3. At day 1, low dose ouabain (5, 10 nM) and oleandrin (50 nM) induced a small increase in proliferation, although other concentrations and digoxin had no effect. In addition, all primary
cultures exhibited increased sensitivity to cardiac glycosides compared to the immortalized cell lines.

Figure 3.7. Cardiac glycosides induce concentration- and time-dependent reductions in proliferation in ER- & PR-positive tumour-derived primary cell cultures.

Cells cultured from ER- & PR-positive, HER2-negative invasive ductal carcinoma were seeded on 96 well plates and grown to 50% confluence before treatment with digoxin, ouabain (5-150 nM) or oleandrin (50-250nM) relative to vehicle control (0.005% DMSO). MTT proliferation assays were performed at 24 and 72 hours. Dose and time dependent effects of digoxin (A), ouabain (B), and oleandrin (C) are shown at one and three days post treatment, as compared to vehicle control (0.0015% DMSO). Treatment with digoxin 40 & 150 nM, ouabain ouabain 5, 40, 100 & 150 nM, and oleandrin 50, 150 & 250 nM reduced
proliferation at 72 hours compared to 24 hours. The graphs show a representative primary culture experiment, with each bar representing two replicates and error bars representing standard deviation. (*p<0.05; **p<0.01; ***p<0.001, compared to control, by Student’s two-tailed unpaired t-test).

Figure 3.8. Cardiac glycosides induce dose- and time-dependent reductions in proliferation in non-tumour epithelial cells from the margins of ER- & PR-positive tumours.

Non-tumour epithelial cells cultured from samples associated with an ER- and PR-positive, HER2-negative invasive ductal carcinoma were seeded on 96 well plates and grown to 50% confluence before treatment with digoxin, ouabain (5-150 nM) or oleandrin (50-250nM) relative to vehicle control (0.005% DMSO). MTT proliferation assays were performed at 24 and 72 hours. Dose and time dependent effects of digoxin (A), ouabain (B), and oleandrin (C) are shown at one and three days post treatment, as compared to vehicle control (0.0015%
DMSO). Significant reductions in proliferation were seen after treatment with digoxin 40 nM, ouabain 10 & 100 nM, and oleandrin 100 nM; at 72 hours compared to 24 hours. Each graph represents the results of experiments on cells derived from three different individuals, while error bars are equivalent to the standard error of the mean. (*p< 0.05; **p <0.01; ***p<0.001, compared to control, by Student’s two-tailed unpaired t-test).
Figure 3.9. Cardiac glycosides induce concentration- and time-dependent reductions in proliferation of primary cultures derived from invasive lobular carcinoma.

Cells cultured from invasive lobular carcinoma were seeded on 96 well plates and grown to 50% confluence before treatment with digoxin, ouabain (5-150 nM) or oleandrin (50-250nM) relative to vehicle control (0.005% DMSO). MTT proliferation assays were performed at 24 and 72 hours. Dose and time dependent effects of digoxin (A), ouabain (B), and oleandrin (C) are shown at one and three days post treatment, as compared to vehicle control (0.0015% DMSO). Treatment with ouabain 100 nM, oleandrin 150 & 500 nM significantly reduced proliferation at 72 hours compared to 24 hours. The graphs show a representative primary culture experiment, with each bar representing two replicates and error bars representing standard deviation. (*p < 0.05; **p < 0.01; ***p<0.001, compared to control, by Student’s two-tailed unpaired t-test).
Figure 3.10. Cardiac glycosides induce concentration- and time-dependent reductions in proliferation in triple-negative tumour-derived primary cell cultures

Cells cultured from ER- and PR-negative, HER2-negative invasive ductal carcinoma were seeded on 96 well plates and grown to 50% confluence before treatment with digoxin, ouabain (5-150 nM) or oleandrin (50-250nM) relative to vehicle control (0.005% DMSO). MTT proliferation assays were performed at 24 and 72 hours. Dose and time dependent effects of digoxin (A), ouabain (B), and oleandrin (C) are shown at one and three days post treatment, as compared to vehicle control (0.0015% DMSO). Significant reductions in proliferation were seen after treatment with digoxin 10 & 40nM, all doses of ouabain, and olaendrin 50, 100 & 250 nM at 72 hours compared to 24 hours. The graphs show a representative primary culture experiment, with each bar representing two replicates and error bars representing standard deviation. (*p<0.05; **p<0.01; ***p<0.001, compared to control, by Student’s two-tailed unpaired t-test).
3.3.2. Determination of the Effects of Cardiac Glycoside Treatment on Breast Cancer Cell Cycle Progression

Having determined that cardiac glycosides exert anti-proliferative effects on breast cancer cell cultures, we sought to establish whether these effects were due to alterations in cell cycle progression, and if so, at which point(s) in the cycle arrest might be induced. To that end, we first performed flow cytometric analysis of MCF 7 and MDA-MB 231 cells treated for 72 hours with digoxin or ouabain (40, 100 nM) compared to vehicle control, and compared the percentage of cells in each stage of the cell cycle. As shown in Figure 3.13, treatment with both cardiac glycosides induced an increase in the proportion of MCF 7 cells in G₁ phase, while all except digoxin 100 nM induced an increase in the proportion of cells in S phase, indicating arrest at both these stages. In contrast, as shown in Figure 3.14, an increase in the proportion of MDA-MB 231 cells in G₁ phase was seen only in those cells treated with digoxin 100 nM (54±3% vs 47±4%), indicating partial arrest of the cell cycle at this stage only after high dose treatment.
Figure 3.11. Cardiac glycoside treatment induces cell cycle arrest at G₁ and S phase in MCF 7 cells.

MCF 7 cells were grown to 50% confluence in dishes before treatment with digoxin (40, 100 nM), ouabain (40, 100 nM) or vehicle control (DMSO 0.001%) for 72 hours. Cells were then trypsinised, fixed and stained with propidium iodide before flow cytometric analysis to determine the percentage of cells in each stage of the cell cycle. Graphs for digoxin and ouabain 100 nM indicate pooled results of two experiments, all others indicate pooled results of three identical experiments. Errors indicate standard error of the mean. Only the increase in the percent of cells in G₁ after treatment with digoxin 40 nM was statistically significant (p<0.01 by Student’s two-tailed unpaired t-test).
Figure 3.12. Cardiac glycoside treatment induces partial arrest of cell cycle in MDA-MB 231 cells.

MDA-MB 231 cells were grown to 50% confluence in dishes before treatment with digoxin (40, 100 nM), ouabain (40, 100 nM) or vehicle control (DMSO 0.001%) for 72 hours. Cells were then trypsinised, fixed and stained with propidium iodide before flow cytometric analysis to determine the percentage of cells in each stage of the cell cycle. Graphs indicate pooled results of three identical experiments. Errors indicate standard error of the mean. Only the increase in the percent of cells in G₁ after treatment with digoxin 100 nM was statistically significant (p<0.01 by Student’s two-tailed unpaired t-test).
Having observed cell cycle arrest at G1 and S phase in MCF 7 cells, and partial arrest at G1 in MDA-MB 231 cells, we next investigated the effects of cardiac glycosides on specific regulators of the cell cycle. As MCF7 cells were most susceptible to cardiac glycoside-induced cell cycle arrest, we subjected this cell line to real time PCR probing for several known positive and negative cell cycle regulating genes. As seen in Figure 3.13, rt-PCR performed on cDNA derived from cells pretreated with digoxin (40 nM for 48 hours) revealed small increases in the M phase regulator cyclin B1; the G1/S phase regulator CDK 2; the G1 phase regulator CDK 4; and the G1/ DNA synthesis regulator p21; in keeping with the observed arrest at G1 and S phase. In contrast, rt-PCR performed on cDNA from ouabain-treated cells (40 nM, 48 hours), revealed a significant increase in p21, with other cell cycle regulators unchanged.
Figure 3.13. Cardiac glycoside treatment induces an increase in p21 gene expression in MCF 7 cells.

MCF 7 cells were grown to 50% confluence and treated with 40 nM digoxin (A), 40 nM ouabain (B), or vehicle control (0.004% DMSO) for 48 hours. RNA was then extracted and converted to cDNA by reverse transcription. cDNA was subjected to real time polymerase chain reaction probing for several positive and negative cell cycle regulators. Each graph represents pooled results of three identical experiments, results indicate fold increase in ΔΔCT compared to vehicle control. (*p < 0.05; **p < 0.01; ***p < 0.001, by Student’s two-tailed unpaired t-test)
Having observed these changes at the gene level, we next examined for changes in cell cycle regulators at the protein level by western blot in both MCF 7 and MDA-MB 231 cells, focusing firstly on p21, as this was the only regulator increased at gene level in response to both digoxin and ouabain treatment. As shown in Figure 3.14, treatment for 72 hours with digoxin and ouabain 40 and 100 nM induced a small increase in p21 expression compared to vehicle control in MCF 7 cells, but not in MDA-MB 231. Since p21 may be regulated by p53, and can work both in a p53-dependent and -independent manner, we examined expression of this protein in both cell lines following exposure to cardiac glycosides. Figure 3.15 shows significant but non-linear reductions in p53 expression in MDA-MB 231 cells after treatment with digoxin, ouabain (40, 100 nM) or oleandrin (100, 250 nM) for 72 hours, compared to vehicle controls. p53 was undetectable in both treated and vehicle control MCF 7-derived lysates.
Figure 3.14. Cardiac glycosides induce a small increase in p21 protein expression in MCF 7 but not MDA-MB 231 cells.

MCF 7 (A,B) and MDA-MB 231 (C,D) cells were grown to 50% confluence in dishes before being treated for 72 hours with digoxin, ouabain (40, 100 nM) or vehicle control (DMSO 0.001%). Cells were then lysed and subjected to SDS-PAGE and Western blot for p21 expression. (A, C) Representative Western blots of MCF 7- and MDA-MB 231-derived lysates, respectively. (B, D) Pooled densitometry results of p21 protein expression, normalized to actin, in three separate replicates of MCF 7- and MDA-MB 231-derived lysates, respectively.
Figure 3.15. Cardiac glycosides induce a decrease in p53 protein expression in MDA-MB 231 but not MCF 7 cells.

MDA-MB 231 cells were grown to 50% confluence in dishes before being treated for 72 hours with digoxin, ouabain (40, 100 nM), oleandrin (100, 250 nM), or vehicle control (DMSO 0.001%). Cells were then lysed and subjected to SDS-PAGE and Western blot for p53 expression. (A) Representative Western blot of MDA-MB 231 derived lysates. (B) Pooled densitometry results of p53 protein expression, normalized to actin, in three separate replicates of MDA-MB 231 derived lysates. p53 protein was undetectable in both treated and control MCF 7 cells (not shown). (*p<0.05; **p<0.01; ***p<0.001, compared to control, by Student’s two-tailed unpaired t-test).
3.3.2. Determination of the Effects of Cardiac Glycoside Treatment on Breast Cancer Cell Migration

Having observed anti-proliferative effects and cell cycle arrest in response to cardiac glycosides in both breast cell lines and primary cell cultures, we considered their effects on cell migration since disordered individual and collective cell migration is a core contributor to malignant behaviour. Many authors have noted similarities between migratory malignant phenotypes and wound healing (90), therefore we used scratch wound assays as a tool to interrogate the impact of cardiac glycosides on cancer cell migration. Specifically, we compared the temporal closure of wounds created by scratching confluent monolayers of MCF 7 and MDA-MB 231 cells following 72 hours pre-treatment with differing concentrations of cardiac glycosides. Cells were photographed immediately after wounding (T0) and six hours later (T6). The six hour time point was chosen as it would allow appreciable wound closure due to cell migration whilst being short enough to exclude a contribution of cell proliferation. Interestingly, 70 nM digoxin in both cell lines induced an increase in wound closure at 6 hours compared to all other concentrations of digoxin, and compared to control in MDA_MB 231 cells. There was a more linear reduction in migration in response to ouabain treatment in both cell lines, and oleandrin in MCF 7, while there was a slight increase in migration seen after low dose (50 nM) oleandrin treatment in MDA-MB 231 cells.
Figure 3.16. Cardiac glycosides inhibit migration in MCF 7 and MDA-MB 231 cells at most doses.

MCF 7 (A-C) and MDA-MB 231 (D-F) cells were seeded in wells of 24 well plates, allowed to adhere overnight and treated with digoxin (10, 70, 120 nM), cuabain (10, 90, 120 nM), oleandrin (50, 150, 250) or vehicle control (0.0012% DMSO). After 72 hours treatment monolayers were wounded and photographed at 0 and 6 hours. Percent wound closure compared to vehicle control was calculated by ImageJ software. (*p<0.05; **p<0.01; ***p<0.001, compared to vehicle control, by Student’s two-tailed unpaired t-test).
3.4. Discussion

The results outlined in this chapter indicate that cardiac glycosides modulate the malignant behaviour of breast cancer cells. Anti-proliferative effects of cardiac glycosides in breast cancer cell lines have been sporadically described in the literature (202-205), however, the majority focus on ouabain, with very few examining the more clinically-relevant drugs digoxin and oleandrin. In addition, only one previous paper has examined the effects of cardiac glycosides on primary breast cancer derived cell cultures (206). Few papers have systematically compared the anti-proliferative effects of different cardiac glycosides in cell lines of differing hormone receptor positivity, and none have examined their effects on cell migration.

In our studies, we focused on the cardiac glycosides digoxin, ouabain and oleandrin. All three compounds broadly inhibited proliferation of both cell lines and primary cell cultures in a dose- and time-dependent manner. However, the triple negative MDA-MB 231 cell line was less sensitive to this inhibition than hormone receptor-positive MCF 7 cells. Furthermore breast primary cell cultures were more sensitive to the effects of cardiac glycosides than the cell lines, and again the triple negative tumour-derived cells were more resistant than hormone receptor-positive cultures. The increased sensitivity of primary tumour-derived cell cultures is encouraging, as they can be considered more representative of a true tumour phenotype than immortalized cell lines. It is an important caveat however that the wide genetic and environmental heterogeneity of patient-
derived material limits the number of true replicates, in turn limiting their broad applicability to mechanistic cell biological investigations. The reduced sensitivity of triple negative breast cancer cells to cardiac glycosides which we observed correlates with the work of other authors (203), and may be due to the suggested anti-oestrogenic effects of cardiac glycosides (170, 207). For example, ouabain is structurally similar to estrogen receptor (ER) ligands such as oestradiol and ethynyl oestradiol. In fact both ligands have been reported to exhibit activity at the Na$^+$ K$^+$ ATPase, respectively stimulating and inhibiting its activity (170, 208). Both the Na$^+$ K$^+$ ATPase and ER$\alpha$ possess similar amino acid motifs that have been hypothesized to bind ouabain (209), and there is some overlap in downstream signalling pathways such as PI3-kinase that both ouabain and oestrogen have been suggested to modulate (210, 211). As ouabain and oestradiol have apparently opposite effects at the Na$^+$ K$^+$ ATPase, and oestradiol plays such a prominent role in breast cancer proliferation (212, 213), this may underpin inhibitory effects of cardiac glycosides on the ER (159). The slight but significant increase in proliferation on treatment with low concentrations of digoxin and ouabain at some time points in MDA-MB 231 cells is of interest, and may perhaps represent pro-proliferative effects at these doses through an as yet undescribed mechanism. However it is noteworthy that cardiac glycosides in general (and digitalis-derived compounds in particular) have a very narrow therapeutic index \textit{in vivo}, and their exertion of effects which are not strictly concentration-dependent highlights a complex receptor pharmacology that remains mechanistically elusive.
In addition to the promising anti-proliferative effects of cardiac glycosides, anti-migratory effects were also observed in response to all three cardiac glycosides in both immortalized cell lines. Other authors have reported both pro- and anti-migratory effects of cardiac glycosides in a cell-specific manner (214-218), although to our knowledge specific anti-migratory effects on breast cancer cells have not been described. Similar to the effects on proliferation, MDA-MB 231 cells appeared less sensitive to the anti-migratory effects of cardiac glycosides than MCF 7 cells, again potentially due to effects at the ER. In support of this, anti-migratory consequences of ER antagonism have been well described in both breast (219) and other malignancies (220, 221), while ER has also been shown to mediate responses to non-oestrogenic ligands such as fibroblast growth factor 2 (222).

Having observed inhibitory effects of cardiac glycosides on breast cancer neoplastic behaviour, we next examined whether, and at what stage, interference with progression through the cell cycle may have contributed to these effects. Flow cytometric analysis of MCF 7 cells pre-treated with cardiac glycosides revealed a relative increase in the percentage of cells in G1 and S phase, and a reduction in the percentage of cells in G2 phase, compared to untreated controls. This may imply a bimodal arrest of cell cycle occurring at both G1/S and S/G2 phase transitions. Other authors have described G0/G1 phase cell cycle arrest in endometrial (223), renal (202) and mesangial cells (224) treated with cardiac glycosides, S phase arrest in hepatoma cells (225), and G2/M phase arrest in leukaemia cells (226). It is interesting however to note that the biphasic cell cycle arrest we observed in MCF 7 cells has not been reported in response to
cardiac glycosides in any other cell type, and may be suggestive of dual effects at both the Na\(^+\) K\(^+\) ATPase and ER. While most published work has shown arrest in G1 in response to ER antagonism (227, 228), the selective oestrogen receptor modulator (SERM) resveratrol has also been reported to cause S phase cell cycle arrest in MCF 7 cells (229), lending credence to this theory. In contrast, similar analysis of ER-negative MDA-MB 231 cells revealed more modest responses to cardiac glycosides, with a significant increase in the proportion of cells in G1 only after treatment with 40 nM ouabain, and a decreased proportion in G2 phase after treatment with 40 and 100 nM ouabain only. This correlates well with the higher relative reductions in proliferation and migration induced by ouabain compared to digoxin in MDA-MB 231 cells and perhaps indicates a higher relative potency. Arrest at G0/1 phase has also been described in other hormone receptor-negative breast cancer cells in response to cardiac glycoside treatment (230). Taken together, our results support the possibility that pure antagonism at the Na\(^+\) K\(^+\) ATPase causes cell cycle arrest at G1 phase, while additional antagonism at the ER in ER-positive breast cancer cells causes additional S phase arrest. This could therefore underlie the particular efficacy of cardiac glycosides in ER-expressing cancers relative to other tumours.

We then examined a range of known cell cycle regulators at both the gene and protein level in cardiac glycoside-treated cells. Real time PCR in MCF 7 cells after treatment with ouabain revealed a 15-fold increase in gene expression of the cell cycle inhibitor p21. In contrast, treatment with digoxin revealed trends towards increased gene expression of p21, CDK 2 and CDK 4 (active during S and G1 phases, respectively); and, surprisingly, the mitosis regulator cyclin B1.
These increases are in keeping with the bimodal G1 and S phase arrest we observed on flow cytometric analysis. Increases in p21 gene expression after cardiac glycoside treatment have been described in hepatoma cells (225), while cardiac glycoside-induced decreases have been observed in renal cyst cells (231). Similarly, seemingly contradictory alterations in p21 levels have been noted in response to both ER stimulation and antagonism: oestradiol has been shown to increase p21 gene activity in MCF 7 cells (232); the ER antagonist tamoxifen has been reported to increase p21 protein expression (233), while overexpressing the ER regulator FKBP1 increases p21 protein expression (234).

At the protein level, Western blot analysis indicated a non-significant increase in p21 expression after treatment of MCF 7 cells with digoxin and ouabain. However this was most marked after treatment with 40 nM digoxin, and declined somewhat after treatment with higher concentrations of digoxin and ouabain. This correlates with the work of several authors who have described increases in p21 protein expression with cardiac glycoside treatment in cell types including hormone receptor-negative breast cancer (235), mesangial tumours (224) and hepatomas (225). In contrast, p21 expression in MDA-MB 231 cells was non-significantly decreased after cardiac glycoside treatment, with the exception of 40 nM digoxin, which induced a non-significant increase. The results outlined here, with increases in p21 expression in ER-positive but not ER-negative cell lines, support that these changes may be due (at least in part) to ER antagonism.

As our results from flow cytometric analysis showed evidence of arrest at the G1/S progression, which is under the influence of the powerful tumour
suppressor p53 (83), itself an activator of p21, we also examined p53 expression in cells treated with cardiac glycosides. Western blot analysis of MDA-MB 231 cell lysates revealed a significant concentration-dependent reduction in p53 expression with all three cardiac glycosides. Since alterations in its gene expression were not detected at the same timepoint (72h) by real time PCR, it is likely that by this time gene expression had normalized while protein changes were only taking effect. Other authors have also observed a decrease in p53 expression after treatment with cardiac glycosides in hormone receptor-negative breast cancer (235) and in lung cancer cells (236). While in lung cancer cells this was accompanied by a predicted decrease in p21 activity (236); a seemingly paradoxical decrease in p53 and increase in p21 expression was noted in MCF 7 cells treated with ouabain. This was ascribed to p21 activation via a p53-independent mechanism, likely involving ERK 1/2 (237). It is interesting to speculate that this alteration in p53 expression may somehow underlie the decrease in sensitivity to cardiac glycosides in MDA-MB 231 cells. It is known that MDA-MB 231 cells express high levels of a mutant p53 (238), and the p53 response to cardiac glycoside treatment may represent an abnormal response to anti-proliferative stimuli in this advanced metastatic cell line. It would be interesting to examine whether forced expression of wild type p53 in MDA-MB 231 cells increases their sensitivity to cardiac glycosides.

We did not detect p53 in Western blots of lysates derived from control or treated MCF 7 cells. This may reflect nuclear localisation of p53 in this cell type (which would be undetected in our study since nuclear material was discarded after centrifugation) or insufficient sensitivity of the antibody used; and more
advanced techniques to isolate nuclear components may be required to detect this protein. Additionally, while MCF 7 cells have been reported to express wild type p53 (237), it is known that there is considerable genetic heterogeneity among MCF 7 populations (191), and even genetically identical populations may exhibit grossly varying behaviours (239). It is possible that our population of MCF 7 cells may have either lost or acquired a mutated p53 that was not detectable by our antibody. Overall however, we speculate that cardiac glycosides may have particular utility as dual antagonists at the Na$^+$ K$^+$ ATPase and the ER in breast cancer expressing the latter, while their effects at the Na$^+$ K$^+$ ATPase alone in ER- negative cells may be of use as adjunctive agents in these cancers.

In conclusion, this chapter has illustrated that cardiac glycosides have potentially powerful anti-neoplastic effects, inhibiting proliferation and migration of both oestrogen receptor- positive and -negative breast cancer cell lines and primary tumour-derived cell cultures. Cardiac glycoside treatment induced an increase in the cell cycle inhibitor p21 at both gene and protein level, causing cell cycle arrest. In addition, cardiac glycoside treatment induced a reduction in p53 expression in hormone receptor-negative MDA-MB 231 cells, which is of uncertain significance but may imply a loss of tumour suppressive ability in this invasive cell line underlying its reduced sensitivity to cardiac glycosides. The following chapter will examine some of the subcellular mechanisms underlying these functional observations.
Chapter 4.

Mechanisms Underlying the Functional Effects of Cardiac Glycosides on Breast Cancer-Derived Cell Lines
4.1. Introduction

The preceding chapter outlined functional effects of the cardiac glycosides digoxin, ouabain and oleandrin on breast cancer-derived cell lines and primary cell cultures, as evidenced by their effects on proliferation, migration and cell cycle arrest. The aim of this chapter was to further examine the mechanisms underlying these effects, with particular attention to the significance of lipid rafts, caveolae and their respective structural proteins flotillin and caveolin. As discussed in chapter 1, caveolin 1 in particular has controversial and seemingly contradictory effects in cancer, being apparently protective against early stage neoplastic growth, but possibly allowing continued tumour cell survival and acquisition of multi drug resistance in advanced cancer (149).

The Na⁺ K⁺ ATPase itself has also been extensively studied with regard to cancer. Several cancers including breast cancer have been shown to highly express the α subunit (203, 214, 240), while its increased expression has been found to correlate with metastasis in melanoma (237) and increased risk of recurrence in bladder carcinoma (237). The α1 subunit in particular has been demonstrated to play a vital role in ouabain-induced cell signalling (237). In contrast, expression of the β subunit has been shown to be diminished in several cancers (237). The β1 isoform has been suggested as a potential tumour suppressor, being reduced in epithelial-to-mesenchymal transition (237), while increased expression has been correlated with extracellular-signal-regulated kinase (ERK) 1/2 activation (237). It is noteworthy however that the vast majority of studies on Na⁺ K⁺ ATPase in cancer have concentrated solely on
gene or protein *expressional* changes, in which a "cause or consequence" relationship with disease has not been clearly established. The idea that altered subcellular localisation of the Na\(^+\) K\(^+\) ATPase might crucially regulate its functions (and thus its contribution to disease) is an under-served area of investigation, and the focus of this thesis.

Likewise, it remains poorly understood how the functional effects of commonly used Na\(^+\) K\(^+\) ATPase antagonists might be influenced by the ability of the protein to localise within distinct membrane compartments. Several studies from the University of Toledo have begun to address the myriad signalling pathways modulated by Na\(^+\) K\(^+\) ATPase in response to binding of ouabain, largely described in cardiac myocytes while researching the pathophysiology of hypertrophy in cardiac failure. For a full description the reader is referred to one of the excellent reviews produced by this group (237). In short, it is postulated that ouabain binding activates the tyrosine kinase Src in caveolae, which in turn complexes with and activates epidermal growth factor receptor (EGFR), activating multiple cascades including the Ras/Raf/MEK/MAPK pathways and increasing production of mitochondrial reactive oxygen species, with the end result of cardiac hypertrophy (237).
4.2. Specific Aims of This Chapter

Having demonstrated functional effects of cardiac glycosides on breast cancer proliferation, migration and progression through the cell cycle, in this chapter we aimed to examine the subcellular mechanisms responsible for these effects. This thesis chapter is particularly interested in the study of lipid rafts and caveolae and their functional relevance to the $\text{Na}^+\text{K}^+$ ATPase in breast cancer. While caveolin has been described as interacting with $\text{Na}^+\text{K}^+$ ATPase in cardiac myocytes, this interaction has never been explored in breast cancer. Given the complex function of caveolin-1 in several malignancies, this association may be of practical use as a target in developing novel anti-cancer therapies.

Our first aim was therefore to examine the impact of cardiac glycoside treatment on the putative presence of $\text{Na}^+\text{K}^+$ ATPase within lipid rafts and caveolae.

Our second aim was to interrogate the respective contributions of the lipid raft and caveolar proteins flotillin-1 and caveolin-1 to the anti-neoplastic functional effects of cardiac glycosides.

As several cell signalling cascades have been described to underlie the functional effects of cardiac glycosides in renal and cardiac cells, but comparatively few researchers have examined these cascades in breast cancer cells; our third aim was to determine whether tyrosine kinase signalling cascades such as Src and MAPK are activated in breast cancer cells in response to cardiac glycosides.
4.3. Results

Having observed varying functional effects of cardiac glycosides in both MCF 7 and MDA-MB 231 cells in chapter 3, and given our interest in the functional relevance of lipid rafts, caveolae and their respective proteins in these effects, we first sought to determine the basal levels of Na\(^+\) K\(^+\) ATPase, caveolin-1 and flotillin-1 in both cell lines. As shown in Figure 4.1, Western blots of lysates derived from untreated MCF 7 and MDA-MB 231 cells showed no significant difference in levels of Na\(^+\) K\(^+\) ATPase \(\alpha_1\) subunit or flotillin-1 between both cell lines, but much higher levels of caveolin-1 expression in MDA-MB 231 cells.
Figure 4.1. MCF 7 and MDA-MB 231 cells differentially express caveolin-1.

MCF 7 and MDA-MB 231 cells were grown to confluence, lysed and subjected to SDS-PAGE to determine expression of Na⁺ K⁺ ATPase α1 subunit, flotillin-1 and caveolin-1. This revealed similar protein expression levels of Na⁺ K⁺ ATPase and flotillin-1 but far greater levels of caveolin 1 in MDA-MB 231 cells. Of note, caveolin-1 is expressed by MCF 7 cells at low levels which are not easily visible at this exposure time. Blot is representative of > 4 separate experiments.
We next investigated the effect of cardiac glycoside treatment on expression of α1 Na⁺ K⁺ ATPase, flotillin-1 and caveolin-1. As shown in Figure 4.2, there was a small increase in expression of α1 Na⁺ K⁺ ATPase in lysates derived from MCF 7 cells pretreated with digoxin and ouabain 100 nM for 72 hours, while that in MDA-MB 231-derived lysates was increased after treatment with ouabain 40 or 100 nM and oleandrin 100 nM, but not after digoxin treatment. Cardiac glycoside treatment did not alter flotillin-1 expression in MCF 7 cells (Figure 4.3), but there was a small reduction in flotillin-1 expression in MDA-MB 231 cells after treatment with oleandrin 100 and 250 nM (Figure 4.4). Caveolin-1 expression appeared to be increased after treatment with 100 nM digoxin and ouabain (Figure 4.3), while its expression in MDA-MB 231 cells was increased after treatment with digoxin 40 or 100 nM, but was not altered in response to any other cardiac glycosides (Figure 4.4).
Figure 4.2. Treatment with cardiac glycosides induces a slight increase in Na⁺ K⁺ ATPase α1 subunit expression.

MCF 7 (A, B) and MDA-MB 231 (C, D) cells were grown to 50% confluence before treatment with cardiac glycosides for 72 hours, followed by lysis and Western blot for Na⁺ K⁺ ATPase α1 subunit expression. Representative Western blots of lysates derived from (A) MCF 7 and (C) MDA-MB 231 cells are shown. (B, C) represent the pooled results of three independent experiments performed on MCF 7 and MDA-MB 231 cells, respectively. There was a small increase in α1 Na⁺ K⁺ ATPase expression after treatment with 100 nM digoxin and ouabain in MCF 7 cells, whilst MDA-MB 231 cells exhibited increased expression after treatment with ouabain 40 or 100 nM and oleandrin 100 nM. (*p<0.05; **p<0.01; ***p<0.001, compared to control, by Student’s two-tailed unpaired t-test).
Figure 4.3. Cardiac glycosides cause a slight increase in caveolin 1 expression in MCF 7 cells.

(A) MCF 7 cells were grown to 50% confluence before treatment with cardiac glycosides for 72 hours, followed by lysis and Western blot for flotillin-1 and caveolin-1 expression. (B) Pooled densitometry of three independent experiments, revealing no significant change in flotillin-1 expression in response to cardiac glycoside exposure. (C) Densitometry on a single representative experiment, showing increased caveolin-1 expression after treatment with 100 nM digoxin and ouabain.
Figure 4.4 Cardiac glycosides induce a decrease in flotillin-1 expression and a slight increase in caveolin-1 expression in MDA-MB 231 cells.

(A) MCF 7 cells were grown to 50% confluence before treatment with cardiac glycosides for 72 hours, followed by lysis and Western blot for flotillin-1 and caveolin-1 expression. (B) Pooled densitometry of three independent experiments, revealing a reduction in flotillin-1 expression after treatment with oleanandrin 100 or 250 nM. (C) Pooled densitometry of three independent experiments, showing increased caveolin-1 expression after treatment with 40 or 100 nM digoxin. (*p<0.05; **p<0.01; ***p<0.001, compared to control, by two-tailed Student’s unpaired t-test)
Having characterized the expression of our proteins of primary interest under basal conditions and in response to cardiac glycoside treatment, our next aim was to biochemically compare their compartmentalisation in the cell membrane relative to lipid rafts, both at baseline and after treatment with cardiac glycosides. We isolated lipid raft fractions via a detergent-free preparation, lysing MCF 7 and MDA-MB 231 cells in sodium carbonate and fractionating in sucrose gradients before analysis by western blot and alkaline phosphatase activity. As shown in Figure 4.5A (MCF 7 cells), peak alkaline phosphatase activity, a defining feature of lipid raft-enriched microdomains, occurred in fractions 6-7 under control conditions (0.0004% DMSO) and following cardiac glycoside treatment (digoxin or ouabain, 40 nM). This suggested no overt disruption or dispersion of lipid rafts in response to cardiac glycoside treatment. In control MCF 7 cells, Na$^{+}$K$^{+}$ ATPase recovery was strongly enriched in a single fraction with high alkaline phosphatase activity (the putative lipid raft pool; Figure 4.5B). There was a striking lack of enrichment of Na$^{+}$K$^{+}$ ATPase in this pool in digoxin- or ouabain-treated MCF 7 cells (Figure 4.5B), suggesting loss of Na$^{+}$K$^{+}$ ATPase affiliation with lipid rafts. Furthermore, while the planar lipid raft marker flotillin-1 was enriched in the fraction corresponding to peak alkaline phosphatase activity in control MCF 7 cells (Figure 4.5C), this focal enrichment was lost following digoxin or ouabain treatment (Figure 4.5C), although it is notable that total flotillin expression appeared to be increased across several fractions after treatment with ouabain, in contrast to the non significant reduction seen in Figure 4.3B. This may perhaps reflect an artefact of the differing lysate preparation methods between the two. Enrichment of caveolin-1 in the fraction
corresponding to peak alkaline phosphatase activity was similar in control MCF
7 cells and those treated with digoxin or ouabain (Figure 4.5D).

MDA-MB 231 cells were next analysed in the same manner (Figure 4.6). Discrete alkaline phosphatase activity peaks were seen in fraction 5 of control MDA-MB 231 gradients, and this was unchanged in response to either digoxin and ouabain (Figure 4.6A). Na⁺K⁺ ATPase recovery was also strongly enriched in this fraction, the putative lipid raft pool, in both ouabain- or digoxin-treated but not control MDA-MB 231 cells (Figure 4.6B). Flotillin-1 recovery in control and digoxin-/ouabain-treated cells coincided with fractions of high alkaline phosphatase activity, but there was no significant difference between that in control and treated conditions (Figure 4.6B). However Caveolin-1 partitioning to putative lipid raft fractions was slightly increased after treatment with cardiac glycosides relative to control conditions (Figure 4.6D).
Figure 4.5. Cardiac glycosides reduce the caveolar partitioning of $\text{Na}^+ \text{ K}^+$ ATPase in MCF 7 cells.

MCF 7 cells were grown to 50% confluence in dishes before treatment with digoxin 40 nM, ouabain 40 nM or vehicle control (0.0004% DMSO) for 72 hours. Cells were then lysed in sodium carbonate based lysis buffer (pH11) and subjected to isopycnic sucrose density gradient fractionation. Fractions were then analysed by alkaline phosphatase assay and Western blot. (A) Alkaline phosphatase activity assay performed on gradient fractions, indicating peak activity in fraction 7 of control and cardiac glycoside treated lysates; corresponding to raft fractions. (B-C) Pooled densitometry of Western blots on gradient fractions for expression of (B) $\text{Na}^+ \text{ K}^+$ ATPase, (C) flotillin-1 and (D) caveolin-1. (B) Caveolar domains were defined by peaks in caveolin-1 expression in fraction 7, correlating with the fraction of peak $\text{Na}^+ \text{ K}^+$ ATPase
expression in untreated MCF 7 lysates. There was some alteration in flotillin-1
distribution following both digoxin and ouabain treatment (C), indicating
possible disruption of planar lipid rafts. However cardiac glycoside treatment did
not appreciably affect the distribution of caveolin-1 (D). (E) Representative
Western blot of vehicle control MCF 7 fractions. Western blot results represent
results of two pooled replicates, while alkaline phosphatase graph represents
three pooled replicates.
Figure 4.6. Cardiac glycosides increase putative raft partitioning of Na⁺ K⁺ ATPase in MDA-MB 231 cells.

MDA-MB 231 cells were grown to 50% confluence before treatment with digoxin 40 nM, ouabain 40 nM or vehicle control (0.0004% DMSO) for 72 hours. Cells were then lysed in sodium carbonate based lysis buffer (pH11) and subjected to isopycnic sucrose density gradient fractionation. Fractions were then analysed by alkaline phosphatase assay and Western blot. (A) Alkaline phosphatase activity of gradient fractions, indicating peak activity in fraction 5 of control and cardiac glycoside-treated lysates; corresponding to raft fractions. (B-C) Pooled densitometry of Western blots on gradient fractions for expression of (B) Na⁺ K⁺ ATPase, (C) flotillin-1 and (D) caveolin-1. (D) Caveolin-1 was expressed discretely in fraction 5-6 in cardiac glycoside-treated lysates, compared to a wider distribution with biphasic peaks in untreated lysates. Na⁺ K⁺ ATPase expression in untreated lysates was also biphasic, with small peaks in
fractions 6 and 9; while that of cardiac glycoside treated lysates peaked sharply in fraction 5 (B). In contrast, there was loss of distinct peak flotillin-1 expression in lysates derived from cardiac glycoside treated cells, compared to control which peaked in fraction 7 (C). (E) Representative Western blot of vehicle control MDA-MB 231 fractions. Western blot results represent results of two pooled replicates, while alkaline phosphatase represents three pooled replicates.

We next attempted to verify the noted effects of cardiac glycosides on caveolar Na\(^+\) K\(^+\) ATPase recruitment *in situ* using triple immunofluorescent staining for Na\(^+\) K\(^+\) ATPase, caveolin-1 and DNA (DAPI) in cells pre-treated for 72 hours with digoxin, ouabain (40, 100 nM), oleandrin (100, 250 nM) or vehicle control (0.001% DMSO). As shown in Figure 4.7D, none of the treatments significantly altered the percentage of Na\(^+\) K\(^+\) ATPase co-localised with caveolin-1, as detected by pixel overlap quantification using ImageJ software analysis. In contrast, in MDA-MB 231 cells (Figure 4.8D), all three cardiac glycosides induced a small concentration-dependent increase in Na\(^+\) K\(^+\) ATPase/caveolin-1 co-localisation. Although the latter did not reach statistical significance, it correlated with our observed biochemical results in this cell line.
Figure 4.7. Cardiac glycosides do not significantly alter Na\(^+\) K+ ATPase/Caveolin 1 co-localisation in MCF 7 cells.

MCF 7 cells pre-treated with cardiac glycosides for 72 hours were fixed and triple-immunolabelled for caveolin-1 (red), Na\(^+\) K+ ATPase (green) and nuclei (DAPI, blue) Areas of co-localisation appear in yellow. Representative images shown: (A) Control; (B) Ouabain 100 nM; (C) Oleandrin 100 nM. (D) Percentage of Na\(^+\) K+ ATPase co-localised with caveolin-1, as quantified using Image J software on pooled images from three separate experiments, revealing no significant alteration after treatment with cardiac glycosides.
Figure 4.8. Cardiac glycosides induce a small increase in Na⁺ K⁺ ATPase/Caveolin 1 co-localisation in MDA-MB 231 cells.

MDA-MB 231 cells pre-treated with cardiac glycosides for 72 hours were triple-immunolabelled for caveolin 1 (red), Na⁺ K⁺ ATPase (green) and nuclei (DAPI, blue). Images show abundant areas of co-localisation (yellow) under all conditions. Representative images shown: (A) Control; (B) Ouabain 100 nM; (C) Oleandrin 100 nM. (D) Percentage of Na⁺ K⁺ ATPase co-localised with caveolin-1, as quantified using Image J software on pooled images from three separate experiments, revealing a trend towards increased overlap in cardiac glycoside-treated cells compared with controls.
Having identified a spatial relationship between Na⁺ K+ ATPase and lipid rafts, and in particular caveolar lipid rafts, and having observed alteration of this relationship upon treatment with cardiac glycosides, we next attempted to identify the potential functional significance of these findings for the anti-neoplastic effects of cardiac glycosides. As we had initially hypothesized that caveolin-1 and caveolar lipid rafts would modulate the anti-neoplastic effects of cardiac glycosides, we first sought to examine whether the loss of caveolin-1 would alter the functional consequences of cardiac glycoside treatment. As shown in Figure 4.9, we successfully silenced caveolin-1 gene expression in MDA-MB 231 cells using siRNA technology, and subjected cells to MTT proliferation assays in the presence of digoxin, ouabain (5, 40, 100 nM), oleandrin (50, 100, 250 nM) or vehicle control (0.001% DMSO). This potentiated the anti-proliferative effects of all cardiac glycosides tested, reaching statistical significance with 100 and 250 nM oleandrin treatment. In contrast, silencing of caveolin-1 in MCF 7 cells (Figure 4.10) did not significantly alter their sensitivity to cardiac glycosides, although the anti-proliferative effects of oleandrin were slightly potentiated. Of note, the anti-proliferative effects of a given agent and concentration on MCF 7 cells is similar to its effect on caveolin-depleted MDA-MB 231 cells in the previous figure.
Figure 4.9. Knockdown of caveolin-1 potentiates the anti-proliferative effects of cardiac glycosides on MDA-MB 231 cells.

MDA-MB 231 cells were seeded in 96 well plates before transfection with non-sense siRNA or siRNA targeted against caveolin-1 in the presence of digoxin, ouabain (5, 40, 100 nM), oleandrin (50, 100, 250 nM) or vehicle control (0.001% DMSO); and subjected to MTT proliferation assays after 48 hours. Caveolin-1 siRNA achieved an approximately 30% average reduction in caveolin-1 expression as quantified by Western blot (A). (B-D) Pooled results of three independent experiments indicated increased sensitivity to the anti-proliferative effects of all three cardiac glycosides in caveolin-knockdown cells compared to nonsense siRNA-treated cells. Proliferation was expressed as percent proliferation relative to that in cells not exposed to cardiac glycosides. (* p< 0.05; **p < 0.01; ***p<0.001, as compared to equivalent dose cardiac glycoside and non-sense RNA, by Student’s two-tailed unpaired t-test).
Figure 4.10. Caveolin knockdown does not significantly alter the antiproliferative effects of cardiac glycosides on MCF 7 cells.

MCF 7 cells were seeded in 96 well plates before transfection with nonsense siRNA or siRNA targeted against caveolin-1 in the presence of digoxin, ouabain (5, 100 nM), oleandrin (50, 250 nM) or vehicle control (0.001% DMSO); and subjected to MTT proliferation assay after 48 hours. (A-C) Pooled results of three independent experiments revealed no significant differences in the antiproliferative effects of cardiac glycosides between nonsense siRNA- and caveolin-siRNA- treated cells. Proliferation was expressed as percent proliferation relative to that in cells not exposed to cardiac glycosides.
Having demonstrated a novel modulating effect of caveolin-1 expression on the anti-proliferative effects of cardiac glycosides in MDA-MB 231 cells, we further sought to test the hypothesis that this occurred via caveolar rather than planar lipid rafts. To this end, we silenced flotillin-1 expression in MCF 7 and MDA-MB 231 cells using siRNA and subjected cells to MTT proliferation assays in the presence of digoxin, ouabain (5, 100 nM), oleandrin (50, 250 nM) or vehicle control (0.001% DMSO). As shown in Figure 4.11 (A) and Figure 4.11 (B), flotillin-1 knockdown did not alter sensitivity to cardiac glycosides in either cell line. This further suggested that caveolar lipid rafts exclusively modulate the anti-neoplastic effects of cardiac glycosides.
Figure 4.11. Flotillin-1 knockdown does not significantly alter the anti-proliferative effects of cardiac glycosides on MCF 7 or MDA-MB 231 cells.

MCF 7 and MDA-MB 231 cells were seeded in 96 well plates before transfection with nonsense siRNA or siRNA targeted against flotillin-1 in the presence of digoxin, ouabain (5, 100 nM), oleandrin (50, 250 nM) or vehicle control (0.001% DMSO); and subjected to MTT proliferation assay after 48 hours. (A, B) Pooled results of three independent experiments in MCF 7 and MDA-MB 231 cells, respectively, revealed no significant difference in the anti-proliferative effects of the cardiac glycosides between the nonsense siRNA- and flotillin-1 siRNA-treated cells. Proliferation was expressed as percent proliferation relative to cells not exposed to cardiac glycosides.
Having identified that caveolin-1 (but not flotillin-1) modulates the functional effects of cardiac glycosides in caveolin-1 expressing breast cancer cells, in addition to evidence of Na⁺ K⁺ ATPase shuttling between raft and non-raft domains in response to cardiac glycoside treatment, we next hypothesised that pharmacological disruption of lipid rafts would also potentiate the anti-proliferative effects of cardiac glycosides in breast cells, while their reconstitution would have the opposite effect. As evidenced in Figure 4.12, this was partially correct. MCF 7 cells were treated with media alone, the raft-disrupting agent methyl β cyclodextrin (MBCD), or cholesterol (delivered using MBCD) to reconstitute lipid rafts; in the presence of digoxin, ouabain (40, 100 nM), oleandrin (100, 250 nM) or vehicle control (0.001% DMSO). As revealed in Figure 4.12 (A), MBCD by itself significantly impaired proliferation in MCF 7 cells, while treatment with exogenous cholesterol partially rescued this. Interestingly, lipid raft disruption abrogated the anti-proliferative effects of both digoxin (Figure 4.12 (B)) and oleandrin (Figure 4.12 (D)). This could not be rescued by raft augmentation with exogenous cholesterol in either case, but it should be noted that the concentration of MBCD used to deliver cholesterol into the cells is the same as that used to abrogate lipid rafts, and likely accounts for the fact that effects observed with the MBCD/cholesterol combination were similar to those with MBCD alone. Raft disruption with MBCD did not alter the anti-proliferative functions of ouabain, but raft augmentation with exogenous cholesterol significantly potentiated its anti-proliferative effects (Figure 4.12 (C)).
In contrast, as shown in Figure 4.13 (A), MBCD by itself significantly impaired proliferation in MDA-MB 231 cells, but treatment with exogenous cholesterol did not rescue this. Lipid raft disruption had no significant effects on the anti-proliferative effects of digoxin (Figure 4.13 (B)), ouabain (figure 4.13 (C)) or oleandrin (Figure 4.13 (D)). In contrast to MCF 7 cells, raft augmentation with exogenous cholesterol abrogated the anti-proliferative effects of all three cardiac glycosides at higher concentrations. This is in keeping with the hypothesis that caveolae may be protective against the anti-neoplastic effects of cardiac glycosides. However, the anti-proliferative effects of cardiac glycosides alone on MDA-MB 231 cells in this experiment were sub-optimal. Taken together, these data further suggest a protective role for lipid rafts in modulating the anti-proliferative effects of cardiac glycosides, although it must be noted that the profound anti-proliferative effects of both MBCD and MBCD/cholesterol alone may perhaps eclipse those of cardiac glycosides.
Figure 4.12. Raft disruption or reconstitution alters the anti-proliferative properties of cardiac glycosides in MCF 7 cells.

MTT proliferation assays were performed on MCF 7 cells in the presence of cardiac glycosides and either cell culture media; 13.2 mM methyl β cyclodextrin (MBCD), which disrupts lipid rafts; or 0.4 mg/mL cholesterol in 13.2 mM MBCD. Cells were seeded in 96 well plates and allowed to adhere overnight; pre-treated with MBCD or MBCD/cholesterol for two hours before replacing with cell culture media containing digoxin, ouabain (40, 100 nM), oleandrin (100, 250 nM) or vehicle control (0.001% DMSO), for 43 hours. At 43 hours the media was replaced again with media containing MBCD or MBCD/cholesterol as well as cardiac glycosides or vehicle control and MTT reagent was added for a further 5 hours before analysis. (A) MTT assays show significant inhibition of proliferation in the presence of MBCD alone, which was partially rescued by the addition of exogenous cholesterol. The Y axis in the graph represents the absolute absorbance at 450 nM, which is directly proportional to cell numbers. (B-D) MTT assays in the presence of cardiac glycosides, revealing that MBCD treatment alone inhibited the anti-proliferative effects of digoxin and oleandrin,
while cholesterol repletion could not restore these effects. Raft disruption with MBCD did not alter the anti-proliferative functions of ouabain, while raft augmentation with exogenous cholesterol significantly potentiated those effects. (* p<0.05; ** p<0.01; *** p<0.001, by Student’s two-tailed unpaired t-test).
Figure 4.13. Raft disruption does not alter the anti-proliferative effects of cardiac glycosides in MDA-MB 231 cells.

MTT proliferation assays were performed on MDA-MB 231 cells in the presence of cardiac glycosides and either cell culture media, 13.2 mM MBCD or 0.4 mg/mL cholesterol in 13.2 mM MBCD. Cells were pretreated with MBCD or MBCD/cholesterol for two hours before replacing with cell culture media containing digoxin, ouabain (40, 100 nM), oleandrin (100, 250 nM) or vehicle control (0.001% DMSO), for 43 hours. At 43 hours the media was replaced again with media containing MBCD or MBCD/cholesterol as well as cardiac glycosides or vehicle control and MTT reagent was added for a further 5 hours before analysis. (A) MTT assays in the absence of cardiac glycosides showed significant inhibition of proliferation in the presence of MBCD, with a slight increase in proliferation induced by the addition of cholesterol. The Y axis in the graph represents the absolute absorbance at 450 nM, which is directly proportional to cell numbers. (B-C) MTT assays in the presence of cardiac glycosides showed no significant change in the efficacy of cardiac glycosides in the presence of MBCD, but a slightly increased resistance to digoxin and
significantly increased resistance to ouabain 40 nM and oleandrin 100 nM with the addition of 0.4 mg exogenous cholesterol. (*p < 0.05; **p < 0.01; ***p < 0.001, by Student’s two-tailed unpaired t-test).

Evidence presented in this thesis so far has highlighted that caveolar lipid rafts play a vital role in modulating the functional outcomes of cardiac glycoside exposure in breast cancer cells, specifically attenuating anti-proliferative effects in highly-invasive triple-negative MDA-MB 231 cells and potentiating anti-proliferative effects in weakly-invasive ER-positive MCF 7 cells. As a final step, we thus turned our attention to candidate cell signalling pathways potentially underlying the effects of cardiac glycosides at the Na⁺K⁺ ATPase, with regard to some of the key messengers which have been implicated in tumourigenic functional behaviours. Since an exhaustive interrogation of all the putative proteins implicated in Na⁺K⁺ ATPase-based signalling would be beyond the scope of this thesis, we concentrated on the early effector and putative ATPase binding partner Src; and the downstream Ras/Raf/MAPK protein ERK 1/2. As shown in Figure 4.14, we subjected MCF 7 and MDA-MB 231 cells to Western blot for total and phosphorylated Src expression after treatment with ouabain (40 nM) or vehicle control for a range of time periods. Ouabain induced a small but statistically significant increase in Src phosphorylation at 5 minutes in MDA-MB 231 cells, while Src phosphorylation in MCF 7 cells peaked at 72 hours after ouabain treatment. In contrast, as shown in Figure 4.15, Western blots for ERK 1/2 phosphorylation revealed that while there was a small transient increase in
ERK 1/2 phosphorylation 5 minutes after treatment in MDA-MB 231 cells, in MCF 7 cells a small increase occurred only 24 hours after treatment. Taken together, these suggest early signalling via Src and ERK 1/2 in MDA-MB 231 cells upon binding of ouabain to Na$^+$ K$^+$-ATPase, while these events are delayed in MCF 7 cells.
Figure 4.14. Ouabain induces increased Src phosphorylation in both MCF 7 and MDA-MB 231 cells.

Cells were grown to >50% confluency in 6 well plates and treated for periods ranging from 1 minute to 72 hrs with ouabain (40 nM) or vehicle control (0.00013% DMSO) before lysis and Western blot for total and phosphorylated ERK 1/2. (A, B) Representative Western blots of lysates derived from respectively MCF 7 and MDA-MB 231 cells. (B, D) Pooled densitometric analysis from two separate Western blots comparing the percentage of active phosphorylated Src, revealing in MCF 7-derived lysates a trend towards bimodal increases in Src phosphorylation after five minutes and 72 hours of exposure to ouabain. In MDA-MB 231-derived lysates there was a small but significant increase in Src phosphorylation after five minutes, with a slight sustained increase at 15 minutes, compared to control. (*p< 0.05; **p <0.01; ***p<0.001, by Student’s two-tailed unpaired t-test).
Figure 4.15. Ouabain causes a small increase in ERK 1/2 phosphorylation in MCF 7 but not MDA-MB 231 cells.

Cells were grown to >50% confluence in 6 well plates and treated for periods ranging from 1 minute to 72 hrs with ouabain (40 nM) or vehicle control (0.00013% DMSO) before lysis and Western blot for total and phosphorylated ERK 1/2. (A, B) Representative Western blots of lysates derived from respectively MCF 7 and MDA-MB 231 cells. (B, D) Pooled densitometric analysis from two separate Western blots comparing the percentage of active phosphorylated extracellular signal-regulated kinase (ERK) 1/2, revealing a trend towards increased ERK 1/2 phosphorylation after 24 hours of exposure to ouabain in MCF 7 cells. In contrast, there was a slight increase in ERK 1/2 phosphorylation in MDA-MB 231 cells 5 minutes after treatment.
4.4. Discussion

The results detailed here provide novel evidence that the powerful anti-breast cancer effects of cardiac glycosides outlined in the previous chapter are modulated by caveolar lipid rafts, via a combination of altered localisation of Na⁺ K⁺ ATPase and caveolin-1, and activation of tyrosine kinase signalling. It is known that the Na⁺ K⁺ ATPase α subunit is overexpressed in several malignancies, and some have suggested that its levels correlate closely with tumour aggressiveness (237). However, our results revealed similar levels of α₁ Na⁺ K⁺ ATPase expression between the weakly-invasive MCF 7 and far more aggressive, highly invasive MDA-MB 231 cells. Further comparison of the levels of α₁ Na⁺ K⁺ ATPase expression between a range of cell lines and primary cell cultures derived from breast tumours of varying aggressiveness and hormone receptor status would be of value in understanding the true importance of absolute levels of this subunit, although, as discussed below, location-dependent functions of Na⁺ K⁺ ATPase may be of more functional relevance than absolute expression. The trend towards a dose-dependent increase in Na⁺ K⁺ ATPase expression in response to digoxin and ouabain treatment is interesting and contrasts with previously published observations of cardiac glycoside-induced downregulation of Na⁺ K⁺ ATPase in renal and breast cancer cells (237). However it is worth noting that, in the case of breast cancer cells, this was only described in relation to lower doses (5-10 nM) of ouabain (237). We speculate that at high doses this may represent a compensatory mechanism in response to inhibition of the Na⁺ K⁺ ATPase pump function (which has been previously
described in renal cells via the PI3K/Akt/mTOR pathway (237)), although one other publication demonstrated no significant pump inhibition in breast cancer cells treated with up to 100 nM ouabain for periods of less than one hour (235). However, as the latter concentration is well in excess of serum levels at which cardiac side effects are seen, and given the long half life and unpredictable pharmacokinetics of digoxin (241), such systemic concentrations of digoxin would be impractical.

There was a large disparity in caveolin-1 expression between the two cell lines; indeed some authors have suggested that MCF 7 cells lack caveolin and caveolae completely (237). It is interesting to speculate that the differing levels of caveolin-1 expression between the two cell lines may influence their differing malignant behaviour. As previously discussed, it has been postulated that loss of caveolin-1 may be an early event in oncogenesis, while later re-expression may facilitate tumour cell survival and malignant spread (153). For example, caveolin-1 null mice do not develop spontaneous tumours but exhibit mammary hyperproliferation and an increased tendency to develop tumours in response to carcinogen exposure (237), suggesting caveolin-1 may be more relevant in the presence of other pro-neoplastic stimuli. Conversely its increased expression correlates with the metastatic phenotype in several cancer including breast (151). Thus it is possible that the MDA-MB 231 cell line represents a later stage in this process, with re-expression of caveolin-1 allowing the development of a highly invasive and metastatic phenotype. This may underlie the much greater tumourigenic potential of the latter in mouse models. The increase in caveolin-1 expression in response to cardiac glycosides is surprising, and to our knowledge
has not been previously reported. While it provides further evidence of a close interaction between Na⁺ K⁺ ATPase and caveolin, the mechanism for this upregulation remains to be discovered but is likely to involve the Na⁺ K⁺ ATPase/caveolin signalling complex.

Given the relatively low level of caveolin-1 and (presumably) caveolae in MCF 7 cells, the fact that flotillin-1 expression was similar between the two cell lines is interesting. As lipid rafts and caveolae are sites for so many vital cellular processes, one might speculate that cells relatively lacking in caveolin-1 might express more planar lipid rafts (and their structural protein flotillin-1) to compensate. It is not known whether and to what extent planar lipid rafts can compensate for the absence of their counterparts, but given the viability of both cells and animals that lack caveolae (237), it seems reasonable to assume that this must occur at least to some extent. This may represent a high degree of redundancy among lipid rafts and caveolae, and a facility to lose high numbers without manifesting significant deficits (although we did not specifically examine for the presence of caveolae per se, other authors have reported that MCF 7 cells have no evidence of caveolae at an ultrastructural level (149)). The downregulation of flotillin-1 expression in response to oleandrin treatment in MDA-MB 231 cells is of uncertain significance, given the mystery currently surrounding the purpose of the flotillins, but it may represent a degree of planar lipid raft disruption. There are however reports that flotillin-1 modulates caveolin-1 expression and prevents its endocytosis (242), which would suggest that its downregulation may lead in turn to downregulation of caveolin-1. Given the effects of caveolin-1 underexpression in MDA-MB 231 cells, this effect may
be of particular relevance in view of the clinicals trials currently underway regarding oleandrin as an adjunctive anti-cancer agent (188).

Interestingly, cardiac glycosides appeared to exert opposite effects on \( \text{Na}^+ \text{K}^+ \) ATPase affiliation with caveolae in the two cell lines tested. In untreated MCF 7 cells, \( \text{Na}^+ \text{K}^+ \) ATPase was highly concentrated in lipid raft fractions isolated by sucrose density gradient fractionation. After treatment with digoxin or ouabain, \( \text{Na}^+ \text{K}^+ \) ATPase was recovered across a broader range of sucrose densities, suggesting reductions in its affiliation with lipid rafts in response to cardiac glycoside treatment. Caveolin-1 distribution was not altered, indicating preservation of caveolae, although flotillin-1 distribution was wider, indicating possible disruption of planar rafts. While there was no significant alteration in \( \text{Na}^+ \text{K}^+ \) ATPase/ caveolin-1 co-localisation detected by immunofluorescence, this may represent a degree of bias induced by the low level of caveolin-1 staining in these cells, as well as sampling limitation due both to the low numbers of cells imaged with immunofluorescence and 2-dimensional imaging of 3-dimensional structures.

In contrast, \( \text{Na}^+ \text{K}^+ \) ATPase recovery in lipid raft fractions was increased after treatment with digoxin and ouabain (compared with control conditions) on sucrose density gradients in MDA-MB 231 cells. Similarly, both caveolin-1 and flotillin-1 demonstrated increased partitioning into raft domains in response to cardiac glycoside treatment. One possible explanation is that cardiac glycosides may actually promote the formation of raft domains, as well as the movement of \( \text{Na}^+ \text{K}^+ \) ATPase into them. Immunofluorescence microscopy in MDA-MB 231
cells also showed a trend towards dose-dependent enhancements in ATPase/caveolin-1 co-localisation in response to cardiac glycosides. These findings support those of the Toledo group, who demonstrated ouabain-dependent enhancements of ATPase/caveolin-1 co-precipitation in renal cells, and surmised that this represented the formation of a signalling complex (237). The disparity between ATPase localisation and subcellular trafficking in MCF 7 and MDA-MB 231 cells echoes other work from our group, which identified altered raft/extra-raft trafficking of CD44 between migrating and non-migrating breast cells (243).

In this context, observed differences in response to caveolin-1 knockdown between MCF 7 and MDA-MB 231 cells could potentially be justified by the relative importance of caveolin-1 to the biological behaviour of the respective cell lines; and the relative importance of Na⁺ K⁺ ATPase and ER-based signalling in their responses to cardiac glycoside treatment. Knockdown of caveolin-1 greatly potentiated the anti-proliferative effects of cardiac glycosides in MDA-MB 231 cells, increasing their sensitivity similar to that of MCF 7 cells. This again illustrates the importance of caveolin re-expression in the survival of advanced malignancies, and the possible role of caveolae in modulating tumorigenic behaviour, and it may also reflect the role of caveolae in function apart from signalling, such as endocytosis of Na⁺ K⁺ ATPase (244). In contrast, caveolin-1 knockdown in MCF 7 cells did not significantly affect their sensitivity to cardiac glycosides. It is likely that, as caveolin-1 is expressed at such low levels in MCF 7 cells, it would play a lesser role in the biology of the cell relative to MDA-MB 231. In addition, as a component of the anti-proliferative
effects of cardiac glycosides may occur via their actions at the ER, these effects would be less influenced by caveolin-1, although of note the ER has also reported to be localised to rafts, where it co-localises with ErbB receptors and is phosphorylated in a MAPK dependent manner (245-247). It is worth noting that multidrug resistant MCF 7 cells acquire increased caveolin-1 expression (149), and that expression of recombinant caveolin-1 in MCF 7 cells has been reported to prevent their detachment-induced apoptosis (anoikis) (237). This would be an interesting area for future research in the context of cardiac glycoside treatment.

Further weight was added to our hypothesis by the observation that flotillin-1 knockdown in both cell lines did not alter the proliferative response to cardiac glycosides in either cell line, and supporting the likelihood that the anti-proliferative properties of cardiac glycosides are modulated by caveolar rather than planar lipid rafts.

Having demonstrated the effects of altering specific lipid rafts by manipulation of their structural proteins, we sought to model whether these were solely due to the action of the proteins in question (eg caveolin-1 & flotillin-1) or might alternatively reflect physical alterations in lipid rafts. To achieve this, we aimed to pharmacologically disrupt and replenish lipid rafts. Methyl β cyclohexextrin (MBCD) is a ring-shaped molecule that acts as a pharmacological tool to remove cholesterol from the cell membrane by sequestering it within the ring structure, disrupting lipid rafts in the process. Interestingly however MBCD is also commonly used as a vehicle to deliver cholesterol and drugs into cells. Cholesterol depletion and disruption of lipid rafts by treatment with MBCD
significantly impaired proliferation in both MCF 7 and MDA-MB 231 cells. Treatment with cholesterol to induce lipid raft formation partially reversed this in MCF 7 but not MDA-MB 231 cells. In varying concentrations MBCD has been described as anti-proliferative and pro-apoptotic in some cell types (237); while at low doses it may have pro-proliferative effects in myoblasts and leukocytes (237). Several authors have described anti-proliferative effects of the cholesterol-lowering agents statins in breast cancer, both in vitro and in vivo (237), which may at least in part be also due to effects on lipid rafts. The concentrations of MBCD and cholesterol used were based on work from our group determined by optimum alteration of transepithelial electrical resistance in CaCo2 intestinal epithelial cells (237) and further optimised based on our own experimentation.

With regard to cardiac glycosides, lipid raft disruption with MBCD appeared to confer some resistance to the anti-proliferative effects of digoxin and oleandrin in MCF 7 but not MDA-MB 231 cells. While this effect in MCF 7 cells must be interpreted cautiously in the setting of profound anti-proliferative effects of MBCD, it correlates with other work indicating that MBCD treatment impairs ouabain-induced signalling (237). Cholesterol repletion slightly increased the sensitivity of MCF 7 cells to ouabain compared to MBCD alone and, in the case of ouabain, compared to control cells. This again correlates with published work indicating a return of ouabain-induced signalling with cholesterol treatment (237). In addition, as a large proportion of ER has been reported to localise in lipid raft domains (237), we speculate that their disruption might impair the anti-
oestrogenic effects of cardiac glycosides on MCF 7 cells, while their reconstitution may partially restore this.

In contrast, treatment with MBCD did not alter the anti-proliferative effects of cardiac glycosides on MDA-MB 231 cells, while cholesterol repletion conferred relative resistance to cardiac glycoside treatment. The latter result correlates well with our results after caveolin-1 knockdown in these cells, again suggesting that intact caveolar lipid rafts confer some immunity to the anti-proliferative effects of cardiac glycosides.

Finally, we interrogated the protein kinase signalling pathways underlying the potential anti-neoplastic effects of cardiac glycosides. As investigating all the putative interlinked cascades implicated in Na\(^+\) K\(^+\) ATPase signalling identified in cardiac and renal cells was impractical, we focussed on Src, as the putative binding partner of Na\(^+\) K\(^+\) ATPase; and ERK 1/2, as a putative downstream effector and part of the versatile Ras/Raf/MAPK cascade. In MDA-MB 231 cells, treatment with 40 nM ouabain induced an increase in activation of the tyrosine kinase Src at 5 minutes, normalizing by 15 minutes, with a smaller increase in ERK 1/2 activation occurring at the same timepoint. Kometiani et al observed similar peaks in Src and ERK 1/2 activation at 5 minutes in ER-negative breast cancer cells (235). In contrast, both Src and ERK activation were seen in MCF 7 cells treated with 40 nM ouabain, although at a much later timepoint. ERK 1/2 phosphorylation peaked at 24 hours post treatment, while Src phosphorylation peaked at 72 hours. This is intriguing considering that ouabain-induced ERK 1/2 activation is postulated to occur downstream of Src, via
activation of EGFR and the Ras/Raf/MAPK cascade (237), and should therefore not precede Src activation. However, it may reflect ERK 1/2 activation through an alternative pathway, perhaps via its effects at the oestrogen receptor.

Indeed, the role of both Src and ERK 1/2 in breast cancer may appear contradictory. The gene encoding Src was initially described as a proto-oncogene (248). Accordingly, Src activation has been shown to promote tumour proliferation, metastasis, angiogenesis and survival (249), while Src inhibitors have shown some promise as anti-breast cancer agents both in vitro (237) and in vivo (250, 251). Similarly, aberrant activation of the Ras/Raf/MAPK pathway, including ERK 1/2, in breast cancer has been associated with increased proliferation, invasion and angiogenesis (252), and overall poorer survival (253), and ERK 1/2 inhibition has yielded some promising anti-neoplastic activity (254). However, our novel findings in this thesis have illustrated Src and ERK 1/2 activation in association with anti-neoplastic signalling. This may have outlined a potential mechanism where Src- and ERK-based signalling crosstalks with as yet unidentified pathways to inhibit proliferation and migration, modulated by the altered Na\(^+\) K\(^+\) ATPase/ caveolin-1 relationship induced by cardiac glycoside treatment. Given that the majority of research on Na\(^+\) K\(^+\) ATPase related signalling has involved either cardiac or renal cells, further work is needed to identify the key upstream regulators and downstream effectors resulting in the seemingly contradictory observation of anti-neoplastic effects in response to cardiac glycoside treatment despite activation of normally pro-neoplastic effectors.
Chapter 5

Discussion
Breast cancer is one of the leading causes of death among Western women. It is both the second most prevalent cancer among Irish women and the second most common cause of cancer mortality (18, 255). Most breast cancers are carcinomas derived from the breast ductal epithelium. Breast cancer aetiology is complex, with several factors including genetic, environmental exposure and hormonal factors all contributing to its development. Mirroring this, the disease itself is complex and heterogeneous with multiple subtypes that can be classified according to hormone receptor, cell of origin, histological grade, clinicopathological stage, or molecular subtype. Treatment is multi-modal, depending both on the above classification systems and patient factors, and usually consists of one or more of surgery, radiotherapy, chemotherapy and hormonal manipulation. Increasingly, therapies targeted against specific tumour markers or antigens have been the focus of much recent attention, with drugs such as the HER2-blocking antibody Trastuzumab already in common clinical use. It is likely that targeted therapies against specific genetic markers represent the future of cancer care, allowing the development of bespoke treatment for most tumours.

The Na⁺ K⁺ ATPase is one protein that has attracted some attention in recent years as a potential anti-tumour target. This membrane-spanning protein primarily functions as an ion channel, extruding three sodium ions in exchange for two potassium ions. However, more recently a secondary role for the Na⁺ K⁺ ATPase has been proposed. Specifically, the existence of a non-pumping pool which functions exclusively in cell signalling has been hypothesized. Interactions
between the Na\(^+\) K\(^+\) ATPase and their naturally-occurring antagonists, cardiac glycosides, have been shown to activate several protein kinase cascades, beginning with the tyrosine kinases Src and Epidermal Growth Factor Receptor (EGFR), and proceeding through mitogen activated protein kinase (MAPK) and several other downstream cascades (256). The net result of these signalling events is tissue-dependent, resulting in increased protein synthesis and hypertrophy in cardiac myocytes (257) and anti-proliferative effects in malignant cells such as breast cancer (170). Several epidemiological papers have noted improved prognosis in patients with breast cancer who have been taking digoxin and related cardiac glycosides for cardiac indications (258-260); and these drugs may be particularly efficacious in breast cancer as they partially antagonise the oestrogen receptor (170). However, some more recent clinical work has suggested that treatment with cardiac glycosides may actually increase the incidence of breast and other hormone-sensitive cancers, by working as ER agonists (261-263). This theory is further evidenced by the reportedly slightly increased risk of ER-positive compared to ER-negative tumours in digoxin users (261). The seemingly contradictory body of evidence here would certainly justify further research.

The quest to understand how the Na\(^+\) K\(^+\) ATPase exercises divergent ion transport-dependent and –independent functions has generated much interest. One possible mechanism relates to sub-cellular compartmentalisation of separate pools of the protein. Accordingly a non-pumping pool of Na\(^+\) K\(^+\) ATPase, the putative target for the anti-proliferative effects of cardiac glycosides, has been reported to reside in membrane organelles known as caveolae, where it interacts
with the structural protein caveolin-1 (167). Caveolae and the caveolins have themselves attracted much interest in cancer research. Caveolae are a subtype of the cholesterol-rich cell membrane microdomains known as lipid rafts, which contain several proteins involved in cell signalling and thus are powerful regulators of numerous cellular processes relevant to neoplastic behaviour (247). Caveolin-1 in particular has been suggested to play a complex role in malignancy; being under-expressed in many early malignancies (suggesting anti-neoplastic effects) while often re-expressed in aggressive or metastatic cancer (suggesting that it promotes metastatic cell survival) (153). While the anti-cancer effects of cardiac glycosides in vitro have been addressed before, the interaction of the Na⁺ K⁺ ATPase pump with caveolin-1 has only been described in cardiac and renal cells. Therefore, given the importance of caveolae and caveolins in cancer development, this thesis sought to systematically interrogate the contribution of caveolae and caveolin-1 to the potential anti-cancer properties of cardiac glycosides in breast cancer cell models.

A selection of three cardiac glycosides was used for these studies based on the following rationale - ouabain as the most commonly used in vivo, digoxin as the most commonly used for cardiac conditions, and oleandrin as the subject of current drug trials for treating certain drug-resistant cancers. As cellular models, we chose two immortalised breast cell lines to represent different phenotypes of breast cancer. MCF 7 is an ER-positive, PR-positive, HER2-negative, weakly invasive cell line (7). In contrast, the MDA-MB 231 cell line is ER-, PR- and HER2-negative and highly invasive (7). To begin translating results from these investigations into a more patient-relevant context, we also performed
representative experiments in a range of primary cell cultures derived from resected breast tumours of varying pathologies.

Firstly, we examined the effects of cardiac glycosides on breast cancer cell proliferation, as previous investigators have reported anti-proliferative effects of cardiac glycosides in both breast cancer and a range of other malignancies (235, 264). ER-positive MCF 7 cells exhibited concentration- and time-dependent reductions in proliferation over three days after treatment with all three cardiac glycosides. In contrast, ER-negative MDA-MB 231 cells were more resistant to cardiac glycosides, with anti-proliferative effects occurring only at higher doses and later time points. Interestingly, pro-proliferative effects were observed with low doses of ouabain in this cell line. Primary tumour-derived cell cultures were in general more sensitive to cardiac glycosides than the cell lines, although we again noted a relative insensitivity in triple-negative primary cultures compared to those derived from ER- and PR-expressing tumours. The greater sensitivity of primary cultures to the anti-proliferative effects of cardiac glycosides (compared with that in immortalised cell lines) is encouraging, as primary cultures more closely represent the tumour environment. However, given the heterogeneity of primary cultures, in vivo studies would be required to truly examine the impact of the complex tumour microenvironment on the functional effects of cardiac glycosides.

Having observed anti-proliferative effects of cardiac glycosides in breast cell lines and primary cultures, we next considered whether they might also impact other functional markers of malignant behaviour. Very little is known regarding
the potential influence of cardiac glycosides on tumour cell migration, a key functional behaviour relevant to the earliest steps of metastasis. While some authors have noted paradoxical effects of ouabain on leukocyte migration (215), only one paper has examined anti-migratory effects of cardiac glycosides in cancer (217). Scratch wound migration assays performed on confluent sheets of MCF 7 cells pretreated with cardiac glycosides for 72 hours revealed significant reductions in wound closure after six hours, while MDA-MB 231 cells were again somewhat insensitive or even registered mild pro-migratory effects in response to low concentrations of oleandrin. Observed increases in migration and proliferation in response to certain low dose cardiac glycosides in MDA-MB 231 cells are intriguing, and may simply be an anomaly related to their low sensitivity. It is tempting to ascribe the non-linearity of anti-proliferative effects, and the impaired sensitivity relative to MCF 7 cells, to the lack of ER expression in these cells; although Kometiani et al reported roughly linear anti-proliferative effects of ouabain on an ER-negative cell line. However, it is notable that their paper used a much wider concentration range to achieve these effects (235). However they may also perhaps represent pro-proliferative and migratory effects via an alternative pathway that remains undiscovered. As they occurred exclusively in the ER-negative cell line, they are not likely to represent agonistic effects at the ER or interactions with oestrogen. To date the majority of published reports examining effects of cardiac glycosides on breast cancer proliferation have focussed on ER-negative cell lines (202, 235, 265); with only three directly comparing the effects on ER-positive and -negative lines. Of these, two observed superior glycoside-mediated inhibition of proliferation in MCF 7 cells (203, 266), while the third reported greatest inhibition in ER-negative
MDA-MB 435 cells (230). While these data raise an interesting conflict, it is noteworthy that MDA-MB 435 cells are less tumourigenic than MDA-MB 231 cells (17). Thus it is intriguing to speculate that the superior ability of cardiac glycosides to modulate neoplastic behaviours in MCF 7 cells may reflect the fact that there is less dysregulation of other relevant signalling pathways relative to MDA-MB 231 cells.

To our knowledge there are no published reports examining the effects of cardiac glycosides on other markers of malignant behaviour in breast cancer such as migration and invasion, both processes necessary for malignant spread. Our data show that cardiac glycosides exert inhibitory effects on collective breast cancer cell migration, while data examining single cell migration (an inherently different process, and perhaps more relevant to metastasis) would be of interest, particularly in poorly-differentiated tumour cells such as MDA-MB 231 that are less likely to migrate collectively (101). Of note, most of the previous work examining cell migration in response to cardiac glycosides has been in cells such as leukocytes that constitutively migrate as single cells (215), while Guo et al, in their paper on gastric carcinoma migration in response to cardiac glycosides, utilised a protocol that primarily examined single cell migration (217). Similarly, while there are reports of cardiac glycosides inhibiting invasion in other cancer cell types (217, 267, 268), our own experiments examining breast cancer cell invasion through artificial basement membranes did not show any significant inhibitory effects of cardiac glycosides (data not shown).
Having examined the effect of cardiac glycosides on functional readouts of malignant behaviour, we next interrogated whether drug-induced alterations in the cell cycle and its regulators could explain the anti-proliferative effects of cardiac glycosides in breast cells. Interestingly, flow cytometric analysis of MCF 7 cells indicated possible bimodal cell cycle arrest occurring at both the G1/S and S/G2 phase transitions after treatment with cardiac glycosides. This bimodal cell cycle arrest is unusual as other authors have only reported cell cycle arrest at a single phase (either G0/1 or S) in response to cardiac glycosides (223-225, 230). This may perhaps represent dual effects at the Na⁺K⁺ ATPase and the ER. Oestrogen antagonism in MCF 7 cells has however been most commonly associated with G1 phase arrest (269, 270), and less commonly with dual arrest in G1 and G2 (271), rather than in S phase as potentially seen here. If this effect is truly due to ER antagonism, it would suggest that this might work via different downstream effectors to those involved with other ER antagonists. In contrast, MDA-MB 231 cells showed less evidence of cell cycle arrest, again in keeping with their relative resistance to cardiac glycosides.

Turning our attention to cell cycle regulators at the gene and protein level, real time PCR revealed increased expression of the cell cycle inhibitor p21 after ouabain treatment in MCF 7 cells, while digoxin treatment induced a smaller increase both in p21 and in regulators of the S, G1 and M phases. Increased p21 expression was also observed at the protein level in MCF 7 lysates following digoxin and ouabain treatment, although in a concentration-independent manner. Interestingly, expression of the cell cycle inhibitor p53 was decreased after treatment with cardiac glycosides in MDA-MB 231 cells, but was undetectable
in glycoside-treated or control MCF 7 cells. Wang et al. reported decreased p53 synthesis in response to cardiac glycoside treatment in MCF 7 but not MDA-MB 231 cells, and further identified that this was via the Src/MAPK pathway (266). In addition, they reported downregulation of p21 protein expression in lung cancer cells in response to treatment with digoxin (266). In contrast, Kometiani et al. reported increased p21 expression after treatment with ouabain in MDA-MB 435 cells (235). One reason for this discrepancy may be the different timepoints used: Wang et al treated for only 24 hours, while Kometiani et al reported peak p21 and lowest p53 levels occurring 48 hours after treatment (235). In comparison, we examined effects occurring after 72 hours of cardiac glycoside exposure. It is possible that 24 hours was insufficient for changes at gene level to be fully translated to protein expression.

Having established functional anti-tumourigenic effects of cardiac glycosides and linked them in part to alterations in the cell cycle, we then focussed on a major theme of this thesis – exploring the impact of cardiac glycosides on the relationship between Na⁺ K⁺ ATPase, caveolae and caveolin-1. There was a large disparity between caveolin-1 expression in the two cell line models selected for study, with very low levels expressed by MCF 7 cells in contrast to MDA-MB 231 cells. This may support the hypothesis that caveolin-1 re-expression in advanced malignancy permits metastasis (147, 151, 152, 272, 273), as MDA-MB 231 cells are highly tumourigenic in mice (274), while MCF 7 cells are weakly tumourigenic and only in the presence of oestrogen treatment (275). We observed a novel increase in caveolin-1 expression in both cell lines after treatment with digoxin, and in MCF 7 cells after treatment with ouabain. The
precise mechanism of how this happens remains elusive, but it is noteworthy that
the cardiac glycoside ouabain has been reported to stimulate overall protein
synthesis in cardiac myocytes (276). In the setting of our results, it further
suggests the importance of caveolin-1 in modulating the anti-proliferative effects
of cardiac glycosides. It is also notable that there is evidence of flotillin-1
modulating caveolin-1 expression in intestinal cells by preventing its degradation
(242), although flotillin-1 expression was unchanged in response to cardiac
glycoside exposure in MCF 7 cells and reduced in MDA-MB 231 after treatment
with oleandrin, a drug that did not alter caveolin expression. In MDA-MB 231
cells, which showed increased ATPase/caveolin-1 co-localisation after exposure
to cardiac glycosides, the increased caveolin-1 expression in response to digoxin
may perhaps represent a feedback mechanism to increase the quantity of
available caveolin-1. Alternatively, as our results also indicated an increase in
\( \text{Na}^+ \text{K}^+ \text{ATP} \alpha_1 \) subunit expression in both cell lines after treatment in cardiac
glycosides, it may reflect the previously observed importance of this subunit in
regulating cavolin-1 membrane trafficking (277). Interestingly, this increase in
\( \text{Na}^+ \text{K}^+ \text{ATP} \alpha_1 \) subunit expression contrasts with other published work which
suggests downregulation in response to ouabain exposure (202).

Previous work from our group has revealed altered raft affiliation of the
membrane adhesion protein CD44 in migrating versus non-migrating breast
cancer cells ((243), Babina et al, personal communication). Given that the non-
pumping pool of \( \text{Na}^+ \text{K}^+ \text{ATP} \alpha_1 \) (implicated in ion transport-independent
signalling) is reported to constitutively co-localise with caveolin-1 in caveolae
(167), we hypothesised that cardiac glycoside treatment might induce the
ATPase to shuttle out of caveolae in a similar manner, altering its interaction with downstream signalling effectors to facilitate anti-neoplastic signalling cascades. This was the case with MCF 7 cells, as determined by detergent free lipid raft extraction and western blot analysis. Intriguingly, the opposite occurred in MDA-MB 231 cells. Wang et al. observed similar effects in renal cells and ascribed this to formation of signalling complexes involving the two proteins (278). This may again reflect the relative importance of caveolin-1 in allowing extra-tumour survival of advanced metastatic cells such as MDA-MB 231 compared to the less-tumourigenic MCF 7 cells.

To further investigate the modulatory effect of caveolin-1 on the anti-neoplastic effects of cardiac glycosides, we reversibly knocked down caveolin-1 and flotillin-1 in both cell lines and subjected them to proliferation assays in the presence of cardiac glycosides. These showed that graded knockdown of caveolin-1 greatly increased the sensitivity of MDA-MB 231 cells to the anti-proliferative effects of cardiac glycosides, while not affecting MCF 7 cells. Furthermore, knockdown of flotillin-1 did not alter the anti-proliferative effects of cardiac glycosides in either cell line. The lack of altered cardiac glycoside sensitivity after flotillin-1 knockdown in both cells adds weight to our hypothesis that it is specifically caveolar rafts that mediate cardiac glycoside-induced anti-proliferative effects.

Having revealed a modulatory role of the raft structural protein caveolin-1, we further sought to examine whether alterations in raft integrity modulated the anti-neoplastic effects of cardiac glycosides. We hypothesized that disruption of lipid
rafts would potentiate the anti-tumoural effects of cardiac glycosides in a similar manner to caveolin knockdown; whilst raft replenishment would have the opposite effect. Interestingly, however, pharmacological disruption of lipid rafts in MCF 7 cells conferred some resistance to the anti-proliferative effects of digoxin and ouabain; while repopulation of rafts by treatment with cholesterol reversed this. This was in keeping with published data in renal cells which showed impairment of ouabain-activated signal transduction after pharmacological lipid raft disruption, which was reversed after cholesterol repopulation (278). In addition, lipid raft disruption may potentially impair effects of cardiac glycosides via the ER, as a large proportion of ER is reported to localise in raft fractions (246, 279).

In MDA MB-231 cells, MBCD treatment did not appear to affect sensitivity to cardiac glycosides. However, the addition of exogenous cholesterol increased resistance to cardiac glycoside treatment; suggesting again that caveolae may have a protective effect on metastatic cell survival. The fact that MBCD did not alter the anti-proliferative effects of cardiac glycosides might imply that lipid rafts are not adequately disrupted in this cell line, and that the Na\(^+\) K\(^+\) ATPase-caveolin-1 interaction is thus unaltered. However, it is worth noting that MBCD itself exerted profound anti-proliferative effects on MDA-MB 231 cells, which were not rescued by raft replenishment with cholesterol. Given this, observed differences between both cell lines in the presence of MBCD and cardiac glycosides may also be related to differential sensitivity to the toxicity of MBCD itself.
Finally, as other authors have postulated the existence of signalling complexes centred on Na\(^+\) K\(^+\) ATPase and caveolin-1, we attempted to elucidate some of the complex signalling pathways underlying the observed effects of cardiac glycosides in both ER positive and negative cell lines, and to indirectly test whether the presence of Na\(^+\) K\(^+\) ATPase in caveolae modulated this signalling. As we know that MDA-MB 231 cells express more caveolin-1 and caveolae, and express a greater proportion of Na\(^+\) K\(^+\) ATPase in caveolae, it is possible that increased pro-tumourigenic signalling occurs as a result of this and underlies the highly aggressive phenotype they display. As a corollary, the presence of such a high proportion of Na\(^+\) K\(^+\) ATPase in caveolae may itself underlie the resistance of this cell line to the anti-proliferative effects of cardiac glycosides, possibly by altering the availability of the ATPase for cardiac glycoside binding. While it has been previously suggested that a large proportion of the functional and signalling effects of cardiac glycosides are due to the non-pumping, raft-affiliated population of Na\(^+\) K\(^+\) ATPase (167), it is possible that bystander inhibition of the extra-raft pool also inhibits ion transport-dependent signalling via other pathways in the cells. Several authors have elegantly described the myriad pathways activated by ouabain treatment in cardiac and renal tissue (256, 280), and to experimentally investigate all these pathways in breast cancer cells would be far beyond the scope of this thesis. We therefore focussed on two major proteins implicated in cardiac glycoside induce signalling; namely Src and ERK. Src is the putative binding partner forming complexes with Na\(^+\) K\(^+\) ATPase and caveolin-1, and can be thought of as the first step in all the ensuing cascades. It is constitutively localised to lipid rafts (281), and its activity is impaired by lipid raft disruption (282) ERK 1/2, as a cornerstone of the Ras/Raf/MAPK pathway,
represents both a downstream effector of Src signalling and a pathway that could potentially be independently activated. In contrast to Src, ERK signalling is less affected by raft disruption (283, 284), suggesting a lesser degree of raft affiliation. Wang et al demonstrated that inhibition of either Src or ERK 1/2 prevents cardiac glycoside-induced modulation of p53 expression in lung cancer cells (266). Both Src and ERK 1/2 have attracted attention as potential pharmacological targets in various cancers, with specific inhibitors at various stages of development (69, 250, 253, 254). We observed transient increases in both Src and ERK 1/2 activation after treatment with ouabain in MDA-MB 231 cells. In MDA-MB 231 cells this occurred at a much earlier timepoint, perhaps due to the increased caveolar affiliation of Na\textsuperscript{+} K\textsuperscript{+} ATPase in this cells line, in addition to increased expression of caveolin-1, the potential binding partner of Src. In contrast, while MCF 7 cells also displayed increased activation of both proteins in response to ouabain, ERK 1/2 activation occurred much earlier than that of Src, suggesting an alternative mode of activation, perhaps via effects at the oestrogen receptor. It is known that ER agonists induce ERK activation, but again this is usually more rapid than observed here (285, 286). The delayed activation of Src in this cell line may also be reflective of the lower proportion of Na\textsuperscript{+} K\textsuperscript{+} ATPase in lipid rafts after cardiac glycoside treatment.

Taken together, our results indicate that cardiac glycosides have significant anti-proliferative and anti-migratory effects in both ER-positive and -negative breast cancer cells, causing cell cycle arrest and increased p21 expression. At the cell membrane, cardiac glycosides act in a caveolin-1-dependent but flotillin-1-independent manner; inducing redistribution of Na\textsuperscript{+} K\textsuperscript{+} ATPase into caveolae in
ER-negative MDA-MB 231 cells, while disrupting this association in ER-positive MCF 7 cells. Enhancement of lipid rafts via cholesterol treatment in MDA-MB 231 cells increases their resistance to cardiac glycosides, with the opposite being true of MCF 7 cells; while cardiac glycoside treatment activates Src- and ERK 1/2-based signalling pathways. However, as Src and ERK 1/2 signalling have themselves been implicated as pro-neoplastic potential targets, it is possible that these signalling events may underlie some of the pro-breast cancer effects that have been recently reported in women taking digitalis preparations (261, 287), and constitute a serious caveat that must be examined before translating this work into a clinical setting.

Taken together, however, our findings suggest that targeting Na\(^+\) K\(^+\) ATPase/caveolin-1 interactions in caveolar lipid rafts may represent a novel opportunity for future anti-breast cancer drug development.

**Future Work**

The work presented in this thesis is novel and raises many questions, suggesting several exciting areas for further research. On a mechanistic level, similar experiments in a range of cell lines would further elucidate the specific impact of hormone receptor positivity, caveolin-1 expression levels or basal tumourigenic potential on the anti-cancer properties of cardiac glycosides. Similarly, expansion of our experimental approach into a larger population of primary tumour-derived cell cultures and eventually *in vivo* patient-derived xenograft murine tumour models would also be valuable. Further work is needed to outline the complex
signalling pathways that we have only begun to elucidate. The potential of cardiac glycosides to act as pharmacological ER modulators (particularly in relation to their superior anti-proliferative effects in ER-positive cells) would be of interest. Another interesting line of enquiry would be to demonstrate whether forced overexpression of caveolin-1 in cells such as MCF 7 would impair their sensitivity to cardiac glycosides. Finally, studies examining the tumours of breast cancer patients concurrently taking cardiac glycosides for cardiac conditions in terms of their grade, staging, expression levels of Na⁺ K⁺ ATPase and caveolin-1; as well as their long term outcomes compared to matched control patients, may yield exciting results and an opportunity to translate this work into the clinical setting.
49. Halperon E, Perez C, LW B. Perez and Brady's Principles and Practice of Radiation Oncology: Lippincott Williams & Wilkins; 2008.
72. Zetterberg A, Larsson O. Kinetic analysis of regulatory events in G1 leading to proliferation or quiescence of Swiss 3T3 cells. Proc Natl Acad Sci U S A. 1985 Aug;82(16);5365-9.

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201. MacLeod RA, Dirks WG, Matsuo Y, Kaufmann M, Milch H, Drexler HG. Widespread intraspecies cross-contamination of human tumor cell lines arising at source. Int J Cancer. 1999 Nov 12;83(4):555-63.


237. .!!! INVALID CITATION !!!


269. Sutherland RL, Hall RE, Taylor IW. Cell proliferation kinetics of MCF-7 human mammary carcinoma cells in culture and effects of tamoxifen on


Appendix
<table>
<thead>
<tr>
<th>Tissue Culture Media</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MCF 7</strong></td>
</tr>
<tr>
<td>MEM</td>
</tr>
<tr>
<td>L-Glutamine</td>
</tr>
<tr>
<td>Non-Essential Amino Acid Solution</td>
</tr>
<tr>
<td>Penicillin</td>
</tr>
<tr>
<td>Streptomycin</td>
</tr>
<tr>
<td>Fetal Bovine Serum</td>
</tr>
</tbody>
</table>

| **MDA-MB 231**               |
| DMEM                         | 500 mL                      |
| L-Glutamine                  | 2 mM                        |
| Penicillin                   | 100 u/mL                    |
| Streptomycin                 | 0.1 mg/mL                   |
| Fetal Bovine Serum           | 10%                         |
# Primary Breast Culture

## Digestion Mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM-F23 Ham</td>
<td>500 mL</td>
</tr>
<tr>
<td>10% Fetal Bovine Serum</td>
<td>50 mL</td>
</tr>
<tr>
<td>10 µg/mL Insulin</td>
<td>5 mL</td>
</tr>
<tr>
<td>5 µg/mL Fungizone</td>
<td>5 mL</td>
</tr>
<tr>
<td>Penicillin/Streptomycin/Neomycin</td>
<td>1%</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>100 U/mL</td>
</tr>
<tr>
<td>Collagenase</td>
<td>200 U/mL</td>
</tr>
</tbody>
</table>

## Feeding Media

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEBM</td>
<td>500 mL</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>1%</td>
</tr>
<tr>
<td>Lonza Bulletkit ®:</td>
<td></td>
</tr>
<tr>
<td>Bovine Pituitary Extract</td>
<td>2 mL</td>
</tr>
<tr>
<td>Human Epidermal Growth Factor</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Gentamicin/Amphotericin</td>
<td>0.5 mL</td>
</tr>
</tbody>
</table>
10X Phosphate Buffered Saline

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>80 g</td>
</tr>
<tr>
<td>KCl</td>
<td>2 g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>14.4 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>2.4 g</td>
</tr>
<tr>
<td>dH₂O</td>
<td>800 mL</td>
</tr>
</tbody>
</table>

Adjust pH to 7.4 with conc HCl
Add dH₂O to 1L final volume

Cardiac Glycosides

Digoxin was dissolved in DMSO to make a 10 mM stock, which was aliquoted and stored at -20°C. Prior to use, aliquots were diluted to 1 μM in the appropriate culture media, sonicated to dissolve them, and stored for up to one week at 4°C before further dilution to the necessary concentration.

Ouabain was dissolved in DMSO to make a 30 mM stock, which was aliquoted and stored at -20°C. Prior to use, aliquots were diluted to 1 μM in the appropriate culture media, sonicated to dissolve them, and stored for up to one week at 4°C before further dilution to the necessary concentration.

Oleandrin was dissolved in PBS to make a 100 μM stock, aliquotted and stored at -20°C. Prior to use, aliquots were thawed and diluted in the appropriate culture media to the required concentration.
Buffers and Reagents Used in Protein Biochemistry

**Relax Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>1.49 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.035 g</td>
</tr>
<tr>
<td>MgCl</td>
<td>0.067 g</td>
</tr>
<tr>
<td>10 mM HEPES</td>
<td>2 mL</td>
</tr>
<tr>
<td>dH₂O to 200 mL</td>
<td></td>
</tr>
<tr>
<td>pH adjusted to 7.4 with HCl/NaOH</td>
<td></td>
</tr>
<tr>
<td>1% each of Triton X 100, protease and phosphatase inhibitor cocktails added just before use</td>
<td></td>
</tr>
</tbody>
</table>

**4X Tris-HCl/SDS Buffer pH 8.8**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trizma Base</td>
<td>90.83 g</td>
</tr>
<tr>
<td>20% SDS</td>
<td>10 mL</td>
</tr>
<tr>
<td>dH₂O</td>
<td>200 mL</td>
</tr>
<tr>
<td>pH adjusted to 8.8 with conc. HCl</td>
<td></td>
</tr>
<tr>
<td>dH₂O to 500 mL total volume</td>
<td></td>
</tr>
</tbody>
</table>

**4X Tris-HCl/SDS Buffer pH 6.8**

<table>
<thead>
<tr>
<th>Component</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Trizma Base</td>
<td>15.14 g</td>
</tr>
<tr>
<td>20% SDS</td>
<td>5 mL</td>
</tr>
<tr>
<td>dH₂O</td>
<td>200 mL</td>
</tr>
<tr>
<td>pH adjusted to 6.8 with conc. HCl</td>
<td></td>
</tr>
<tr>
<td>dH₂O to 250 mL total volume</td>
<td></td>
</tr>
</tbody>
</table>
10% Ammonium Persulphate

Ammonium Persulphate 1 g
dH₂O 10 mL

Aliquotted and stored at -20°C

SDS-PAGE Gels

Separating Gel

<table>
<thead>
<tr>
<th>Chemical</th>
<th>8% Gel</th>
<th>10% Gel</th>
<th>12% Gel</th>
</tr>
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<tbody>
<tr>
<td>30% Acrylamide/ 0.8% Bisacrylamide</td>
<td>4 mL</td>
<td>5 mL</td>
<td>6 mL</td>
</tr>
<tr>
<td>Tris-HCl/SDS pH 8.8</td>
<td>3.75 mL</td>
<td>3.75 mL</td>
<td>3.75 mL</td>
</tr>
<tr>
<td>dH₂O</td>
<td>7.25 mL</td>
<td>6.25 mL</td>
<td>5.25 mL</td>
</tr>
<tr>
<td>10% Ammonium Persulphate</td>
<td>50 µL</td>
<td>50 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µL</td>
<td>10 µL</td>
<td>10 µL</td>
</tr>
</tbody>
</table>

Stacking Gel

30% Acrylamide/ 0.8% Bisacrylamide 0.65 mL
Tris-HCl/SDS pH 6.8 1.25 mL
dH₂O 3.05 mL
10% Ammonium Persulphate 25 µL
TEMED 5 µL
4X Laemmli Sample Buffer

SDS 0.5 g
Tris HCl pH 6.8 5 mL
Glycerol 4 mL
dH₂O 1 mL
Dithiothreitol (DTT) 800 μL
Bromophenol blue to achieve desired colour
Stored at -20°C

10X Tris-Glycine

Trizma Base 30.3 g
Glycine 144 g
dH₂O to 1L final volume

10X Trizma Buffered Saline (TBS)

NaCl 80 g
KCl 2 g
Trizma Base 30 g
dH₂O 800 mL
pH adjusted to 7.4 with conc. HCl
dH₂O to 1L final volume
1X TBS

100 mL 10X TBS diluted to 1 L with dH₂O

1X TBS-Tween

100 mL 10X TBS diluted to 1 L with dH₂O and 1 mL Tween 20 added

Running Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trizma Base</td>
<td>3.03 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>14.4 g</td>
</tr>
<tr>
<td>20% SDS</td>
<td>5 mL</td>
</tr>
</tbody>
</table>

dH₂O to 1 L final volume

Transfer Buffer

<table>
<thead>
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<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trizma Base</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>11.5 g</td>
</tr>
<tr>
<td>Methanol</td>
<td>160 mL</td>
</tr>
</tbody>
</table>

dH₂O to 800 mL final volume

Ponceau Red

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ponceau S</td>
<td>50 mg</td>
</tr>
<tr>
<td>dH₂O</td>
<td>50 mL</td>
</tr>
<tr>
<td>Glacial Acetic Acid</td>
<td>150 μL</td>
</tr>
</tbody>
</table>
**Stripping Buffer**

Tris-HCl/SDS pH 8.8 50 mL
20% SDS 39 mL
dH₂O to 400 mL

Just before use, 70 μL β-Mercaptoethanol added to 10 mL for each membrane to be stripped.

**Hanks Balanced Salt Solution (HBSS) for Lipid Raft Isolation**

1 bottle HBSS without sodium bicarbonate and phenol red (Sigma) was dissolved in 900 mL dH₂O
0.35 g sodium bicarbonate was added
10 mL 1 M HEPES was added
pH was adjusted to 7.4 using HCl/NaOH

This was combined 1:1 with 500 mM Na₂CO₃ and used to prepare 90%, 30%, 20% and 5% (w/v) sucrose solutions. Sucrose was dissolved by heating and vortexing and cooled to 4°C before use

**Alkaline Phosphatase Assay Reagent**

pNPP tablet and Tris buffer tablet warmed to room temperature
Both tablets added to 5 mL dH₂O and vortexed to dissolve

Used within one hour
### Chemicals and Suppliers

<table>
<thead>
<tr>
<th>Reagent</th>
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<th>Product Code</th>
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</thead>
<tbody>
<tr>
<td>Acrylamide/ Bis-acrylamide</td>
<td>Sigma</td>
<td>A3699</td>
</tr>
<tr>
<td>Ammonium persulfate</td>
<td>Sigma</td>
<td>A3678</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>Sigma</td>
<td>A7906</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>Sigma</td>
<td>B8026</td>
</tr>
<tr>
<td>BCA Protein assay kit</td>
<td>Pierce</td>
<td>23227</td>
</tr>
<tr>
<td>Caveolin-1 siRNA</td>
<td>Thermo Scientific</td>
<td>M-003467-01</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Sigma</td>
<td>C3045</td>
</tr>
<tr>
<td>DAPI</td>
<td>Sigma</td>
<td>D9163</td>
</tr>
<tr>
<td>Digoxin</td>
<td>Sigma</td>
<td>D6003</td>
</tr>
<tr>
<td>DMED</td>
<td>Sigma</td>
<td>D6546</td>
</tr>
<tr>
<td>DMEM/Ham’s F12</td>
<td>Sigma</td>
<td>D8437</td>
</tr>
<tr>
<td>DMSO</td>
<td>Sigma</td>
<td>D5879</td>
</tr>
<tr>
<td>DTT</td>
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195
| Mouse anti Flotillin-1 | 1:1000 | BD Biosciences | 610820 |
Publications

(Attached at rear of thesis)

Manuscripts Published

McSherry, EA, Owens MB, Hopkins AM

Lipid rafts are disrupted in mildly inflamed intestinal microenvironments without overt disruption of the epithelial barrier.
PMID: 22241861

Under Review

Owens MB, Hill ADK, Hopkins AM.
**Ducal Barriers in Mammary Epithelium**, invited review in Ductal Barriers, special issue of Tissue Barriers, submitted April 2013.

In Preparation

Owens MB, Brennan K, Hudson L, Hill ADK, Hopkins AM.
**Cardiac Glycosides Inhibit Breast Cancer Proliferation and Migration Via Altered Caveolin 1- Na⁺K⁺ ATPase Interactions**
Presentations


Owens MB, Hill ADK, Hopkins AM. Cardiac Glycosides Inhibit Breast Cancer Proliferation and Migration. Oral presentation. SARS, University of Nottingham, January 2012.

Owens MB, Hill ADK, Hopkins AM. Cardiac Glycosides Inhibit Breast
Cancer Proliferation and Migration. Poster presentation. Trinity
International Cancer Conference, Trinity College, September 2011.

Owens MB, Hill ADK, Hopkins AM. Cardiac Glycosides, Caveolae and Breast

Owens MB, Hill ADK, Hopkins AM. Cardiac Glycosides as Potential Anti-
Cancer Agents. Poster presentation. Irish Association for Cancer Research,
March 2011.

Owens MB, Hill ADK, Hopkins AM. Cardiac Glycosides as Potential Anti-
Cancer Agents. Poster presentation. Shepherd Prize, Beaumont Hospital,
February 2011.
Lipid rafts are disrupted in mildly inflamed intestinal microenvironments without overt disruption of the epithelial barrier

Rachel V. Bowie,1* Simona Donatello,1* Cliona Lyes,1* Mark B. Owens,1 Irina S. Babina,1 Lance Hudson,2 Shaun V. Walsh,2 Diarmuid P. O'Donoghue,3 Sylvie Amu,4 Sean P. Barry,4 Padraic G. Fallon,4 and Ann M. Hopkins1
1Department of Surgery, Royal College of Surgeons in Ireland; 2Department of Pathology, Ninewells Hospital, Dundee, Scotland; 3Centre for Colorectal Disease, St. Vincent’s University Hospital, Dublin; and 4Institute of Molecular Medicine, St. James’s Hospital, Trinity College Dublin, Ireland

Submitted 7 January 2011; accepted in final form 10 January 2012

mation of the epithelial barrier is a feature of inflammatory bowel disease (IBD), but whether barrier disruption preceeds or merely accompanies inflammation remains controversial. Tight junction (TJ) adhesion complexes control epithelial barrier integrity. Since some TJs proteins reside in cholesterol-enriched regions of the cell membrane termed lipid rafts, we sought to elucidate the relationship between rafts and intestinal epithelial barrier function. Lipid rafts were isolated from Caco-2 intestinal epithelial cells primed with the proinflamma-
tory cytokine interferon-γ (IFN-γ) or treated with methyl β-cyclodextrin as a positive control for raft disruption. Rafts were also isolated from the ilea of mice in which colitis had been induced in conjunction with in vivo intestinal permeability measurements, and lastly from intestinal biopsies of ulcerative colitis (UC) patients with predomin-
nantly mild or quiescent disease. Raft distribution was analyzed by measuring activity of the raft-associated enzymes alkaline phosphatase and by performing Western blot analysis for flotillin-1. Epithelial barrier integrity was estimated by measuring transepithelial resistance in cytokine-treated cells or in vivo permeability to fluorescent dextran in colitic mice. Raft and nonraft fractions were analyzed by Western blotting for the TJ proteins occludin and zonula occludens-1 (ZO-1). Our results revealed that lipid rafts were disrupted in IFN-γ-treated cells, in the ilea of mice with subclinical colitis, and in UC patients with quiescent inflammation. This was not associated with a clear pattern of occludin or ZO-1 relocation from raft to nonraft fractions. Significantly, a time-course study in colitic mice revealed that disruption of lipid rafts preceded the onset of increased intestinal permeability. Our data suggest for the first time that lipid raft disruption occurs early in the inflammatory cascade in murine and human colitis and, we speculate, may contribute to subsequent disruption of epithelial barrier function.

epithelium; barrier function; tight junction; colitis; adhesion

THE INTESTINAL EPITHELIAL barrier separates the contents of the gut lumen from the interstitium. Barrier function is maintained via protein-protein interactions between neighboring cells at adhesion sites called tight junctions (TJs). The dynamic intercellular seal formed by TJs creates a polarized semipermeable barrier across which the traffic of nutrients and antigenic particles is tightly regulated (31, 33). Accordingly, the integrity of the intestinal epithelial barrier is a crucial determinant of mucosal homeostasis (39), and impaired epithelial barrier function has been implicated in mucosal inflammatory disorders including Crohn’s disease and ulcerative colitis (UC) (35, 48).

It remains controversial whether epithelial barrier dysfunction is a primary cause or merely a consequence of inflammatory bowel disease (IBD). In vivo patient evidence has argued both for (15, 17, 34) and against (41, 54) a role for altered intestinal permeability in the etiology of IBD. Recent work in mouse models has echoed the conflict, with reports that permeability alterations precede the onset of colitis in IL-10 knockout mice (29, 47), whereas loss of barrier function alone is insufficient to induce murine colitis in mice constitutively overexpressing myosin light chain kinase in the intestinal epithelium (52). Intermodulation should serve to illustrate the complexity of the problem and to highlight that much remains to be understood about the regulation of epithelial adhesion complexes and barrier function during intestinal inflammation.

Some key TJ proteins reside in subdomains of the plasma membrane known as detergent-resistant membranes or lipid rafts (42). Lipid rafts are unique biological entities enriched in glycosphingolipids, cholesterol, and sphingomyelin, giving them a "liquid-ordered" state resulting from tighter packing of these lipids relative to the "liquid-disordered" state of phospholipids in the bulk plasma membrane (4, 13). Raft localization of some TJ proteins involves cholesterol-mediated stabilization within the membrane (20). Accordingly, interference with cellular cholesterol levels can profoundly impact TJ assembly and the establishment of barrier function (11, 21, 25-27).

However, little is known about the potential regulatory influence of lipid rafts on epithelial adhesion and barrier function under inflammatory conditions. Displacement of the TJ protein junction adhesion molecule-A from raft fractions has been noted in intestinal epithelial cells exposed to a combination of the proinflammatory cytokines IFN-γ and TNF-α (6), and the same cytokines have been shown to alter the lipid composition of membrane microdomains (22). Therefore, our study investigated the relationship between raft integrity, barrier function, and an inflammatory microenvironment using three complementary models: intestinal epithelial cells primed with a proinflammatory barrier-disrupting cytokine, mouse models of colitis, and human intestinal IBD biopsies. We focused on experimental conditions best described as early inflammatory or primed for inflammation, to dissect events that might precede epithelial barrier disruption rather than accom-
BARRIER DISRUPTION AND LIPID RAFTS

Table 1. Profiles of biopsy patients in pilot study

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Biopsy Histopathology</th>
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<tbody>
<tr>
<td>Control 1</td>
<td>Female</td>
<td>61</td>
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<td>UC 3</td>
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UC, ulcerative colitis.

Table 2. Profiles of biopsy patients in secondary study

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<td>UC 12</td>
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MATERIALS AND METHODS

Cell culture and treatments. Caco-2 cells were obtained from ATCC and maintained in DMEM plus Glutamax, containing 4,500 mg/l d-glucose (Invitrogen, Paisley, UK) supplemented with 10% (vol/vol) fetal bovine serum (Lonza, Basel, Switzerland), 1% (vol/vol) MEM nonessential amino acids (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cultures were subcultured with 0.05% trypsin-EDTA (Invitrogen) and grown either on 0.33-cm² polyester Transwell filters (0.4 μm pores, Costar) or on 10-cm dishes. Once filters reached a high stable transmucosal resistance (measured using an EndOhm-6; World Precision Instruments), they were treated for 7 days with the anti-inflammatory cytokine interleukin-10 (IL-10, 10 ng/ml; R&D Systems) or for 5 days with the proinflammatory cytokine interferon-γ (IFN-γ, 150 U/ml; R&D Systems). In combination experiments, cells were incubated with IFN-γ and IL-10 for 5 days following 2 days of pretreatment with IL-10. Control cells were treated with vehicle alone (sterile water/0.1% bovine serum albumin; Sigma). As a positive control for lipid raft disruption, some filters were treated bilaterally for 30 min with 10 mM methyl-β-cyclodextrin (MBβCD, Sigma) and some dishes were treated for 3 h with 3 mM MBβCD.

Preparation of intestinal mucosal lysates from colitic mice. Intestinal tissues were excised from two separate mouse models of colitis in parallel with ilea from age- and sex-matched control animals. In the first model, 8- to 10-wk-old C57BL/6 mice were treated for 3 days with 2% dextran sulfate salts (DSS; MP Biomedicals, OH) in drinking water to initiate colitis (10, 50). In complementary experiments, mice were treated with DSS for either 0, 3, or 5 days and assessed in parallel for intestinal permeability (see Mouse intestinal in vivo permeability assay) or lipid raft status. The second model used 10- to 12-wk-old Balb/c mice genetically engineered to lack the IL-10 gene, in which colitis spontaneously develops from 10 to 12 wk (10, 19). The ileum was opened along the mesenteric border and irrigated in ice-cold HBSS/10 mM HEPES to remove the luminal contents. Tissues were washed and agitated for 5 min in Ca²⁺/Mg²⁺-free isolation buffer (30 mM EDTA, 52 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM DTT, and 60 mM HCl, pH 7.1) (24), then minced and agitated for 2 x 10 min in isolation buffer at 4°C. Released cells were collected by centrifugation at 100 g for 10 min at 4°C. Mucosal isolates from DSS mice were resuspended in HBSS/10 mM HEPES containing 1% Triton X-100 and a protease inhibitor cocktail for lipid raft isolation. The nondenaturing Triton X-100 was used in preference to stronger ionic detergents on the basis of the definition of lipid rafts as insoluble in cold nonionic detergents (38). Mucosal isolates from IL-10 knockout animals were resuspended in 500 mM sodium carbonate (pH 11) containing a protease inhibitor cocktail for detergent-free lipid raft isolation. In all cases, two ilea from each group were pooled per raft preparation. All animal experiments were performed in compliance with regulations issued by the Irish Department of Health and Children and were approved by the Trinity College BioResources ethical review board.

Mouse intestinal in vivo permeability assay. In vivo intestinal permeability to fluoresceinated dextran was assessed essentially as described (53). Briefly, mice exposed to 2% DSS for either 0, 3, or 5 days (2 per condition, n = 3 experiments) were orally gavaged with 0.6 mg/g body wt of fluorescein isothiocyanate (FITC)- labeled dextran (4 KDa, TDB Consultancy, Uppsala, Sweden). After 3.5 h the mice were euthanized and exsanguinated by cardiac puncture. Plasma FITC levels were subsequently determined by fluorometry.

Isolation of intestinal mucosal homogenates from human patient biopsies. In a pilot study, superficial mucosal biopsies were obtained from six patients attending a gastroenterology outpatient clinic: three controls and three UC patients (Table 1). Specimens were obtained with informed consent after full ethical approval had been granted by the Ethics and Medical Research Committee of St. Vincent’s University Hospital, Dublin, Ireland. Control samples were obtained from healthy patients undergoing colonoscopy screening for cancer. Areas were sampled on the basis of macroscopically visible inflammation or the macroscopic appearance of healthy, normal tissue. Histopathological reports were later used to confirm the official diagnosis. Biopsies were immersed in ~2.5 ml ice-cold HBSS/HEPES containing 1% Triton X-100 and a protease inhibitor cocktail and mechanically disaggregated on ice with 35 strokes of a Dounce homogenizer followed by 15 passes through a 26-gauge needle. Lysates were rotated for 30 min at 4°C before being mixed with 60% sucrose for lipid raft preparation as described in Lipid raft isolation from Caco-2 cells and DSS mouse mucosal lysates.

We subsequently extended the study by obtaining mucosal biopsy specimens from 17 patients using the same methods and under the same ethical approval as above. Patient demographics are reported in Table 2 and in brief amounted to 5 control patients and 12 UC patients (of which 7 had inactive disease, 3 had active disease, and a further 2 had active disease but were biopsied in a macroscopically uninvolved area distal from the disease). Biopsies were transported, homogenized, rotated, and mixed with 60% sucrose exactly as above, but a slightly different procedure was used for isolation of lipid rafts as described below.
BARRIER DISRUPTION AND LIPOP RaFTS

Lipid raft isolation from Caco-2 cells and DDS mouse mucosal l�ysters. Isotypic sucrose density gradient fractionation was used to isolate lipid rafts as described (42). Briefly, confluent Caco-2 cells (2 × 10^6 cells) were harvested from BD Biosciences (2 × 10^6 cells) or mucosal l�ysters prepared from DDS and control animals were washed × 3 in ice-cold HBSS-10 mM HEPES and dounced × 20 in HBSS-HEPES-1% Triton X-100 containing a protease inhibitor cocktail. Then 2 ml l�ysters was combined with 2 ml 60% (w/v) sucrose and transferred to a 12.5-ml ultracentrifuge tube (Beckman Coulter, Galway, Ireland). This was overlaid with a linear 20–5% sucrose gradient and ultracentrifuged for 19 h (39,000 rpm, 4°C) in a Beckman Optima L-100K ultracentrifuge (SW41 rotor). Gradients were fractionated by use of a model 2110 Fraction Collector (Bio-Rad, Hemel Hempstead, UK), ensuring that any cloudy bands were collected in a single fraction. Pellets were solubilized in 500 μl 0.5% SDS (Sigma).

Detergent-free lipid raft isolation from mucosal lыysters of IL-10 knockout mice. Mucosal lыysters from IL-10 knockout mice or age- and sex-matched wild-type mice were resuspended in 500 mM sodium carbonate (pH 11) containing a protease inhibitor cocktail, dounced and triturated 20 times, and rotated at 4°C for 20 min. Lыysters were mixed with an equivalent volume of 90% sucrose (in 250 mM sodium carbonate pH 11), and 740 μl were placed at the bottom of a microcentrifuge tube. The 490 μl volumes of 30, 20, and 5% sucrose (all prepared in 250 mM sodium carbonate pH 11) were sequentially layered on top and tubes were ultracentrifuged for 19 h (52,000 rpm, 4°C) in a Sorvall M210EX microcentrifuge (RP55SS rotor). Six equal-volume fractions were removed, and the pellet was resuspended in the same volume of 0.5% SDS before being analyzed as described in Analysis of Lipid raft gradient fractions.

Lipid raft isolation from human mucosal homogenates. For our pilot study, human mucosal homogenates were combined with equivalent volumes of 60% sucrose (prepared in HBSS/10 mM HEPES), of which 240 μl was placed at the bottom of a microcentrifuge tube and layered sequentially with 490 μl of 50% sucrose, 20% sucrose, and finally 5% sucrose. Gradients were ultracentrifuged in a Sorvall M210EX microcentrifuge and fractionated into six equal fractions plus an SDS-soluylized pellet.

For the larger population of 17 patient samples, full-size continuous sucrose gradients of 12-ml volume were poured exactly as described for the Caco-2 and DDS preparations and centrifuged as described in a Beckman SW41 rotor before being fractionated into 12 equal-volume fractions and analyzed as above.

Analysis of lipid raft gradient fractions. Sucrose density was read in 2-μl volumes of all fractions via a hand-held refractometer (Bellingham-Stanley). Raft-enriched fractions were identified on the basis of a peak in activity of the raft-associated enzyme alkaline phosphatase. Briefly, 20-μl fractions were incubated with p-nitrophenyl phosphate substrate (Sigma, St. Louis, MO) at 37°C for at least 1 h. Yellow coloration, indicative of alkaline phosphatase activity, was quantitated at 405 nm on a Wallac Victor 1420 plate reader (Perkin Elmer). Protein concentrations in each fraction were determined by the bicinchoninic acid method (Pierce), relative to a standard curve of bovine serum albumin.

Electrophoresis and Western blotting. Representative raft and non-raft fractions from sucrose gradients were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes under constant voltage and Western blotted as described (16) for the lipid raft marker flotillin-1 and the TJ proteins occludin and zonula occludens-1 (ZO-1). Equivalent protein concentrations (5–40 μg) were run for human gradients, and equivalent volumes were run for gradients from cytokine-treated cells and mice samples. Antibodies were obtained from BD Biosciences (flotillin-1 mouse IgG), Invitrogen (occludin and ZO-1 rabbit IgGs), and Sigma (horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG secondaries). Signals were detected by enhanced chemiluminescence using Western Lightning (Perkin-Elmer) and Kodak film.

Immunofluorescence confocal microscopy. Caco-2 cells grown to confluence on permeable supports (as described above) were treated for 2 h with 10 mM MβCD or vehicle (sterile water), then fixed in paraformaldehyde (3.75%; 10 min) and permeabilized with Triton X-100 (0.5%; 30 min). Cells were blocked in goat serum (5%; h) and stained for F-actin and the nuclei with Alexa Fluor 488 phalloidin (0.3 μM; h) followed by DAPI (1 μg/ml; 10 min). Filters were mounted in p-phenylene diamine hydrochloride-PBS-glycerol (1:1:1 vol/vol/vol) antifade reagent and examined on a Zeiss LSM 510 Meta confocal microscope.

Statistical analysis. Quantitative data are expressed as means ± SE, with n numbers and internal replicates indicated in the figure legends. Statistical analysis was done by two-sample equal-variance Student’s t-tests, with significance assumed at P < 0.05.

RESULTS

Mild reductions in intestinal epithelial cell barrier function are accompanied by lipid raft disruption. Epithelial barrier function was evaluated by the measurement of transepithelial electrical resistance (TER) in confluent, polarized Caco-2 intestinal epithelial cells that had been exposed to combinations of the proinflammatory cytokine IFN-γ (to disrupt barrier function) and the anti-inflammatory cytokine IL-10 as a control reagent that should not damage barrier function. As shown in Fig. 1A, TER of control (vehicle-treated) cells at day 7 was ~100% of the day 0 value. Similarly, IL-10 treatment for 7 days did not alter baseline TER values. However, exposure to IFN-γ for 5 days caused a subtle but statistically significant reduction in TER, to 70 ± 10% of baseline values. Transepithelial permeation of the paracellular marker fluorescein isothiocyanate dextran (3,000 Da) was also increased in IFN-γ-treated cells relative to controls (data not shown). Cells that had been preexposed to IL-10 for 2 days followed by 5 days of coexposure to IL-10 and IFN-γ also showed reductions in TER that were comparable with those induced by IFN-γ alone (to 66 ± 10% of baseline values by day 7). In contrast, treatment of Caco-2 cells with the lipid raft disrupting agent MβCD for just 30 min virtually abrogated TER. The dramatic compromise in intestinal epithelial barrier function induced by MβCD could not be accounted for by necrotic cell death (as estimated by MTT assay), since the viability of Caco-2 cells treated with 10 mM MβCD was >97% even after 3-h exposure (Supplemental Fig. S3A). There was also no evidence of death by apoptosis secondary to MβCD treatment, as confirmed by the absence of cleaved caspase-3 staining in Caco-2 cells treated for 30 min with 10 mM MβCD, relative to a positive control of etoposide (200 μM for 24 h; Supplemental Fig. S3B). However, cellular polarity was disrupted by MβCD treatment, with evidence of multilayering in xz confocal micrographs of treated relative to control cells (Fig. 1A, inset).

Since barrier function is regulated by membrane proteins in the TJ, some of which have been shown to affiliate with lipid rafts (42), we next tested whether the mild reductions in barrier function featured disruption of membrane lipid rafts. Isotypic density gradient centrifugation was used to isolate lipid rafts from Caco-2 monolayers treated with the cytokine combinations described above, or with 3 mM MβCD to induce raft disruption. Figure 1B represents the profiles of fractions isolated from sucrose gradients; the left panel shows an equivalent (predictable) increase in sucrose density with increasing fraction number across all gradients. Similarly, the protein concentration across all gradients was similar (middle), being very
Fig. 1. Lipid rafts are disrupted in Caco-2 intestinal epithelial monolayers primed with the proinflammatory cytokine interferon-γ (IFN-γ). A: filter-grown confluent Caco-2 intestinal epithelial monolayers were exposed bilaterally to interleukin-10 (IL-10; 10 ng/ml; 7 days) + IFN-γ (150 U/ml; days 3–7) or vehicle, and posttreatment transepithelial electrical resistances (TER) expressed as % of pretreatment values. Both IFN-γ and IL-10 + IFN-γ significantly reduced Caco-2 TER, but to a lesser extent than the lipid raft-disrupting reagent methyl-β-cyclodextrin (MβCD; 10 mM; 30 min). The graph represents means ± SE of 4 independent experiments, with ≥2 filters/experiment (**P < 0.01; ***P < 0.001). Inset: in xz confocal micrographs of cells treated with MβCD (10 mM; 2 h) vs. vehicle control (con), it was seen that MβCD treatment disrupted cell polarity. B: confluent Caco-2 cells on 10-cm tissue culture dishes were exposed to IL-10 (10 ng/ml; 7 days) + IFN-γ (150 U/ml; days 2–7) or vehicle. Lipid rafts were isolated by sucrose density gradient fractionation, and fractions analyzed for sucrose density, protein concentration and activity of the raft marker enzyme alkaline phosphatase. Alkaline phosphatase (Aphos.) activity was enriched in a single fraction from control preparations, whereas in IFN-γ- or MβCD-treated preparations it was spread across 2–3 fractions with approximately equal density. Graphs represent means ± SE of 3 independent experiments. C: gradient fractions as above were blotted for the lipid raft marker protein flothlin-1. Flothlin-1 was strongly enriched in a single fraction from control cells, but spread equally across several fractions in cells treated with IFN-γ + IL-10 or MβCD. *Lipid raft fractions compared with control gradients.
dilute in earlier fractions and most concentrated in the higher fraction numbers (≥30% sucrose density).

Alkaline phosphatase activity (Fig. 1B, right) was used as an indicator of the presence of rafts, due to the enrichment of this membrane enzyme in lipid rafts (38). A single peak of alkaline phosphatase activity was observed in fraction 4 of control samples (and fraction 5 of IL-10-treated samples), suggesting that rafts were strongly enriched in a single fraction (~22–26% sucrose). In contrast, gradients isolated from cells treated with IFN-γ (alone or in combination with IL-10) showed equivalent alkaline phosphatase activity across multiple fractions, not peaking in a single one. A similarly broad distribution was observed in MβCD-treated cells, suggesting that both treatments disrupted lipid rafts and prevented their predominant recovery from a single gradient fraction. To further test this, equivalent volumes of sequential gradient fractions were probed by Western blotting for the lipid raft marker flotillin-1 (Fig. 1C). Although protein concentrations in the lower fractions (fractions 1-3) were negligible compared with those in the higher fractions (fractions 6-9), flotillin-1 was strongly enriched in a single fraction (fraction 4) in both control and IL-10 gradients. In contrast, flotillin-1 was equally spread across fractions 3-6 in gradients from IFN-γ-treated cells and MβCD-treated cells. Lack of flotillin-1 enrichment in a single fraction of either cytokine-treated and MβCD-treated cells suggested similar levels of raft disruption, although only MβCD treatment caused a significant depletion of cholesterol from Caco-2 cells whereas IFN-γ treatment did not (data not shown).

Since the disruption of Caco-2 barrier function obtained with IFN-γ was relatively modest, we also exposed filter-grown Caco-2 cells to concentrations of IFN-γ ranging from 50 to 1,000 U/mL. As shown in Supplemental Fig. S1A, IFN-γ evoked concentration-dependent reductions in TER, which after 5 days exposure were statistically different from controls at concentrations ≥150 U/mL (the concentration used in our studies). A time-dependent component to TER reductions induced by IFN-γ was also evident (Supplemental Fig. S1B). Specifically, whereas TER of control filters and those treated with the lowest concentration of IFN-γ (50 U/mL) remained stable or increased over 7 days, TER of filters treated with IFN-γ concentrations ≥150 U/mL gradually decreased after 3 days' exposure and plateaued at 5–7 days.

To begin interrogating potential links between IFN-induced reductions in barrier function and lipid raft status, in a proof-of-principle experiment we performed isopycnic density gradient fractionation on Caco-2 cells treated for 7 days with each of the indicated concentrations of IFN-γ. As shown in Supplemental Fig. S2A, the profiles of fractions isolated from each condition were relatively similar in terms of sucrose density (left), protein concentrations (middle), and alkaline phosphatase intensity (right). However, densitometric quantification of expression of the lipid raft marker flotillin-1 in each of the gradient fractions (represented per unit protein concentration; Supplemental Fig. S2B) revealed flotillin expression to be highest in raft fraction 5 of control conditions, with less flotillin expression in the same fraction of all IFN-treated conditions. Another difference was that flotillin expression dropped very sharply in fraction 6 of controls but remained in two adjacent fractions of all IFN-treated conditions (suggesting a potential dispersal of rafts). Finally, analysis of occludin expression in equivalent volume fractions (Supplemental Fig. S2C) revealed enriched recovery in fraction 5 of control conditions (in light of the very low protein concentration in this sample) and also high levels recovered at the highest sucrose densities at the bottom of the gradient. Strikingly, occludin was recovered additionally in fractions 3 and 4 of all IFN-treated gradients (corresponding to a very low sucrose density of only 11–18% at which occludin is never normally recovered). This provides suggestive evidence of raft dispersal or disruption in a concentration-independent fashion in response to IFN-γ treatment even at concentrations before barrier disruption is evident.

Lipid rafts are disrupted in two mouse models of colitis even without histological evidence of inflammation. Having demonstrated that lipid raft disruption was associated with mild epithelial barrier dysfunction in intestinal epithelial cells primed with the proinflammatory cytokine IFN-γ, we next wanted to determine whether lipid raft disruption was associated with in vivo intestinal inflammation. We first utilized a DSS mouse model of colitis, in which mice were exposed to 2% DSS for 3 days. Lipid rafts could not be successfully isolated from the colon (data not shown) even at this early stage when the DSS-treated mice had not yet developed overt colitis-like symptoms [as assessed via a disease activity index score including weight loss, presence of diarrhea, and blood in feces (50)]. Therefore we harvested the ilea for lipid raft isolation, because, although the ileum of DSS mice traditionally is histologically uninfamed, it is nonetheless within an inflammatory microenvironment in the intestine as a whole. As shown in Fig. 2A, hematoxylin- and eosin-stained sections taken through the proximal, mid, and distal ileum and examined by a histopathologist (S. V. Walsh) confirmed no histological differences between 3-day DSS-treated and control mice. In particular, no evidence of cryptitis, crypt abscess, ulceration or excess chronic inflammatory cells was seen in the DSS animals. Villi remained tall and slender, mitotic cells were confined to the basal compartment of the crypts, and there was no change in Paneth cell or goblet cell numbers.

Epithelial lysates harvested from control and DSS mice ilea were then subjected to sucrose density gradient fractionation, whereupon characterization of the gradient fractions revealed sucrose density and protein concentration profiles between treatment groups (Fig. 2B, left and middle, respectively). The only exception was that protein concentration dropped off in the higher sucrose density fractions of DSS samples relative to controls.

When lipid raft recovery was analyzed in DSS relative to control gradients, a peak of alkaline phosphatase activity was observed in a single fraction of control preparations (Fig. 2B, right), suggesting a high density of rafts harvested from control preparations. Alkaline phosphatase activity did not peak in a single fraction of IFN-γ- or MβCD-treated preparations but was recorded at approximately equivalent levels across two to three fractions. When equivalent fraction volumes were immunoblotted for flotillin-1, enrichment of the raft marker in a single fraction of control gradients was observed (Fig. 2C). It is interesting to note that the raft-enriched fraction, fraction 4, had a total protein content of approximately four-to-fivefold less than that of fractions 8 and 9, which contained no detectable flotillin-1. In contrast to control gradients, flotillin-1 expression in DSS gradients was not enriched in a single fraction.
Fig. 2. Small intestinal lipid rafts are disrupted in mucosal preparations from mice with mild colitis. A: hematoxylin/ eosin-stained small intestinal mucosal sections of C57BL/6 mice exposed for 3 days to 2% dextran sulfate sodium (DSS) or control mice. No evidence of inflammation was observed in DSS sections relative to controls. B: Lipid rafts were isolated from the ileal mucosa of C57BL/6 mice exposed for 3 days to 2% DSS or control mice, and gradient fractions were analyzed for sucrose density, protein concentration, and activity of the raft marker enzyme alkaline phosphatase. Peak alkaline phosphatase activity was reduced in fractions from DSS mice. Graphs represent means ± SE of 3 independent gradients (2 mice per gradient). C: gradient fractions as above were blotted for the lipid raft marker protein flotillin-1. The distribution of flotillin-1 was broader in DSS-treated mice fractions relative to controls. *Lipid raft fraction. D: lipid rafts were isolated from the ileal mucosa of IL-10 knockout mice and wild-type controls by using a detergent-free method of sucrose density gradient fractionation. Equivalent volumes of gradient fractions were electrophoretically separated and blotted for flotillin-1. A broader distribution of flotillin-1 was observed in IL-10 knockout gradients relative to controls. P, pellet.

but rather recovered at equivalent band densities across multiple fractions. This suggested a disruption of lipid rafts rather than a global increase in flotillin-1 expression levels, since total flotillin-1 levels in control and DSS lysates were similar to each other (data not shown).

To exclude the possibility that lipid raft disruption in the uninfamed ilea of DSS mice was artifactual, we utilized a second mouse model and an alternative lipid raft isolation strategy. Lipid rafts were isolated from the ilea of mice genetically deficient in IL-10, in which mild low-grade inflammation...
had developed in the colon. Ileal inflammation in these animals is possible but rare, and in our animals no ileal inflammation was observed at a histological level (data not shown). When detergent-free lipid raft gradients were prepared and analyzed by Western blotting, we detected strong enrichment of flotillin-1 in a single fraction of control gradients (Fig. 2D). In contrast, flotillin-1 bands were dispersed among several gradient fractions prepared from IL-10 knockout mice, being enriched in high sucrose density fractions (>32%) rather than the 22–26% sucrose density fractions in which rafts are characteristically found. Taken together with our previous observations, this supported the hypothesis that lipid raft disruption occurs even in uninfamed/uninvolved areas of the intestine within an inflammatory intestinal microenvironment.

To better interrogate the temporal relationship between loss of barrier function and putative lipid raft disruption, we performed a time course of 2% DSS treatments in C57/B6 mice and examined in vivo permeability in parallel with lipid raft status. As shown in Fig. 3A, intestinal permeability to the paracellular tracer FITC-dextran (4 KDa) was low in animals treated for either 0 or 3 days with DSS. This indicates no significant loss of intestinal epithelial barrier function by 3 days, the time frame used for the experiments described above. In contrast, mice exposed to the same concentration of FITC-dextran for 5 days showed significant loss of barrier function, with over fivefold more tracer detected in plasma ($P < 0.001$; n = 3 experiments).

When lipid raft gradients were poured from ileal epithelial preparations of mice treated for 0, 3 or 5 days with DSS, the sucrose density profiles and protein concentrations were quite similar between all conditions (data not shown). However, subtle differences were observed in the profile of alkaline phosphatase activity across fractions from different gradient conditions (Fig. 3B). Enzyme activity peaked on a single fraction (fraction 6; corresponding to 22.3 ± 1.7% sucrose density) in control/0 days DSS animals. In contrast, enzyme activity in 3-day DSS preparations was slightly lower and did not peak in a single fraction. Instead relatively equivalent levels of enzyme activity were observed across fractions 4–6; corresponding to 17.4 ± 1.1 to 24.5 ± 0.8% sucrose. This suggested mild disruption of lipid rafts in 3-day DSS animals (consistent with our data in Fig. 2), and it is interesting to note that lipid rafts are never usually recovered below 20% sucrose. Finally, two abnormally high peaks of alkaline phosphatase intensity were observed in fractions 5 and 6 of gradients prepared from animals treated for 5 days with DSS, corresponding to 21.3 ± 0.3 and 24.5 ± 0.2% sucrose. When gradients were blotted for the lipid raft marker flotillin-1 (representative blots shown in Fig. 3C), there was generally enrichment of flotillin-1 in one to two fractions of control (0 days DSS) preparations, whereas the profile was broader in both 3- and 5-day DSS preparations. No particular differences could be seen between the broadened profiles of 3- and 5-day preparations, suggesting that any raft disruption had already occurred by the early time point and did not worsen over time.

UC patient intestinal biopsies display evidence of lipid raft disruption. To translate the preceding results into the context of human inflammation, we undertook a pilot study to analyze lipid raft status in a small group of UC patients with mild or quiescent inflammation relative to control patients with no history of IBD. As shown in Fig. 4A (left), there was no difference between the sucrose densities in gradient fractions from either control or UC patients (UC 3 and control 3 shown; see Table 1). Protein concentration profiles (co) were broadly similar (Fig. 4A, middle), although slightly less protein was
Fig. 4. Lipid rafts are disrupted in human biopsy homogenates from ulcerative colitis (UC) patients. A: lipid rafts were isolated by density gradient fractionation from intestinal biopsy homogenates of a control patient (control 3) and a patient with mild ulcerative colitis (UC 3). Gradient fractions were analyzed for sucrose density, protein concentration and activity of the lipid raft marker enzyme alkaline phosphatase. Peak fraction intensity for alkaline phosphatase activity was reduced and shifted to a lower fraction in the UC sample relative to control. B: equivalent concentrations of gradient fractions (5 μg/lane control; 10 μg/lane UC) were electrophoretically separated and Western blotted for the raft marker flotillin-1. Enrichment of flotillin-1 was observed in lipid raft-associated fractions (3 and 4) from the control gradient, but only in fractions at the bottom of the gradient prepared from UC homogenates (fractions 5 and 6). C: in a separate study involving 17 patients (5 controls and 12 UC), we prepared sucrose density gradients and compared alkaline phosphatase activity levels between fractions from the different conditions. Enzyme activity peaked in fraction 6 of control samples and then declined in the following fractions before increasing again in the protein-rich fractions at the bottom of the gradient (fractions 9–12). In contrast, enzyme activity was similar in fractions 6–12 of UC samples and did not decline before reaching the bottom of the gradient.

recovered from the higher gradient fractions of UC vs. control tissues. This was echoed by a lower protein concentration in mucosal homogenates from UC vs. control samples and may reflect mucosal demudulation in patients with prior inflammation.

When alkaline phosphatase enzyme activity assays were performed to identify the gradient fractions putatively containing lipid rafts (Fig. 4A, right), interesting differences between control and UC samples were observed. Specifically, a high peak of activity was observed in fraction 3 (~23% sucrose) of the representative control sample, whereas a peak of lower intensity was observed in fraction 2 (~15% sucrose) of the representative UC sample. Analysis of equivalent protein concentrations (5 μg/lane) in control fractions by SDS-PAGE and Western blot analysis (Fig. 4B) revealed enrichment of the raft marker flotillin-1 in the predicted raft fraction, fraction 3, along with fraction 4. In contrast, equivalent protein concentrations electrophoretically separated from the UC gradient (10 μg/lane) detected flotillin-1 only at the bottom of the gradient, at >32% sucrose density in fractions 5 and 6. Consistent with our previous results, this indicated a possible disruption of lipid
rafts in even uninvolved regions within an inflammatory microenvironment.

In a larger study, we also used density gradient fractionation to isolate lipid rafts from 17 patient biopsy samples (Table 2). Five and 12 samples represented, respectively, control and UC conditions, with only 2 of the 12 UC samples having active inflammation. Analysis of the 12-raft gradients for sucrose density profiles, as expected, revealed no differences between control and UC conditions (data not shown). Similarly, there were no real differences between the protein content profiles of control vs. UC gradients (data not shown). Alkaline phosphatase activity profiles of control gradients (pooled in Fig. 4C) revealed a single small peak in enzyme activity in fraction 6, corresponding to 22.5 ± 0.3% sucrose, the density at which lipid rafts are normally recovered. As expected, there was also a predictable rise in alkaline phosphatase activity toward the bottom of the gradient (fractions 9–12), reflecting the much higher protein concentrations in later fractions. In contrast, pooled samples from UC gradients did not show a single peak of alkaline phosphatase activity in fraction 6. Instead there was a small and approximately equivalent peak in enzyme activity across fractions 6–8 (23.5 to 30.7 ± 0.1% sucrose), suggesting lipid raft disruption or dispersal. As with control samples, alkaline phosphatase activity was also high in the high-protein fractions 9–12 at the bottom of the gradient.

Because of exceedingly low protein concentrations in individual fractions, the lipid raft marker flotillin-1 could not consistently be detected by immunoblots even after acetone precipitations were carried out to concentrate the volumes (data not shown). However, on the basis of extensive experience that flotillin-1 expression is typically highest in the middle fractions (21–25% sucrose), showing a peak of alkaline phosphatase activity, we thought it likely that flotillin-1 expression would be highest in a single fraction of control samples (fraction 6) but more diffusely spread out across fractions 6–8, reflecting disrupted lipid rafts within UC gradients.

Affiliation of TJ proteins with lipid rafts is mildly perturbed during early inflammation. Having presented evidence of lipid raft disruption in cells, mouse tissues, and human biopsy samples under pre-infl ammatory conditions featuring only minor (if any) disruptions in barrier function, we wanted to test whether this reflected altered affiliations between candidate TJ proteins and lipid rafts. Accordingly, raft vs. nonraft fractions from cytokine-treated Caco-2 cells were Western blotted for the raft-associated TJ protein occludin (Fig. 5A). Occludin was strongly enriched in raft fractions (fractions 4 and 5) relative to nonraft fractions (fractions 10 and 12) in control monolayers. Lysate levels of occludin were reduced in all cytokine-treated conditions, and there was a small increase in the levels of occludin recovered in nonraft fractions from IFN-γ or IL-10-treated cells in parallel with reductions in occludin recovery from raft fractions.

When equivalent protein concentrations of raft vs. nonraft fractions from mouse gradients were blotted for occludin (Fig. 5B, top), we observed occludin enrichment in raft fractions from control animals. Mucosal lysate levels of occludin were substantially reduced in DSS-treated animals, and occludin was not detected in raft or nonraft subcellular fractions. This is unlikely to reflect a global loss of protein in DSS samples since no histological sloughing of the epithelium was observed (Fig. 1A), total protein profiles were similar (Fig. 1B), and overall flotillin-1 expression was not altered even by 5-day DSS treatment (Supplemental Fig. S4). Fractions were also blotted for the raft-associated TJ protein, ZO-1. As shown (Fig. 5B, bottom), ZO-1 levels were slightly reduced in mucosal lysates of DSS relative to control animals. On a subcellular level, ZO-1 was recovered predominantly in the raft fractions of both control and DSS animals.

Lastly, the profile of occludin expression in raft vs. nonraft fractions was determined in gradients isolated from human
intestinal biopsy homogenates (Fig. 5C). Data from UC 3 and control 3 are represented here (for patient details see Table 1). Because of the global reduction in protein expression previously noted in UC relative to control samples (Fig. 4B), 10 and 5 µg/mg of tissue were loaded in, respectively, UC and control gradients. As shown in the representative control sample in Fig. 5C, occludin was predominantly recovered in raft-associated fractions 3 and 4. In contrast, occludin was only recovered in nonraft fractions prepared from a UC patient with mild inflammation (fractions 5 and 6). Overall levels of occludin protein were also reduced in the UC sample.

**DISCUSSION**

An intact intestinal epithelial barrier is essential for homeostasis. Loss of barrier function can result in self-amplifying cycles of immune activation, facilitating uncontrolled transepithelial passage of antigens (9). Epithelial disruption has been linked to inflammation in intestinal disorders including IBD. TJ proteins, key regulators of barrier function, have been associated with cholesterol-enriched membrane domains termed lipid rafts (20, 42). Thus the aim of this study was to investigate links between rafts and barrier dysfunction in pre-/early-inflammatory microenvironments without overt inflammation by using a cell line, two mouse colitis models, and human IBD biopsies.

In accordance with published data (11, 21, 25–27), the raft-disrupting agent MβCD impeded both formation (data not shown) and maintenance of epithelial barrier function. Exposure of Caco-2 intestinal epithelial cells to MβCD induced rapid reductions in TER paralleled by enhanced paracellular permeability (data not shown). To simulate inflammation, cells were treated with the proinflammatory cytokine IFN-γ, which induces barrier leakiness (1, 28, 36, 58). Under our conditions, IFN-γ provoked mild barrier function reductions compared with those induced by MβCD. Barrier function was unaltered by the control cytokine IL-10, nor could the reported anti-inflammatory properties of IL-10 protect against the barrier-disruptive effects of IFN-γ.

We questioned whether mild barrier compromise induced by IFN-γ was associated with disruption of lipid rafts, since several barrier-regulatory TJ proteins reside in rafts. Rafts were separated from Caco-2 lysates on the basis of a characteristic low buoyant density after ultracentrifugation on sucrose gradients. Immunoblotting for the raft marker flotillin-1 revealed a broader distribution in IFN-γ or MβCD gradients relative to controls, consistent with raft dispersion. Raft disruption in response to IFN-γ or MβCD was further supported by a wider distribution of alkaline phosphatase enzyme activity across several gradient fractions, relative to control samples where an activity peak was detected in a single fraction (∼22–26% sucrose). However, since TER reductions were much greater with MβCD than IFN-γ (despite similarities in raft disruption), this may imply a complex relationship between raft disruption and barrier disruption. Treatment of T84 intestinal epithelial cells with IFN-γ and TNF-α has been shown to alter the lipid composition of lipid rafts in conjunction with loss of barrier function (22). Thus it is possible that the fundamental organization of rafts is impacted in inflammatory microenvironments, which in turn could influence the localization of key membrane proteins and the execution of their biological functions (adhesion/signaling).

Cells primed with proinflammatory cytokines lack the complexity of in vivo models. IFN-γ is a key contributor to inflammation in murine models of colitis, particularly in the DSS model to which IFN-γ-null animals are resistant (18). Thus we examined the link between rafts and barrier integrity in mice primed for intestinal inflammation by low doses of DSS. The DSS model reliably reproduces several features of UC (44), and under our conditions small intestinal involvement was histologically undetectable. Although we could not isolate colonic lipid rafts in our system because of very low yields of colonic epithelial cells (even from as many as 4 mice pooled per condition), we found that even ideal lipid rafts were disrupted, evidenced by broadened flotillin-1 distribution and reductions in peak alkaline phosphatase activity in DSS animal levels of lipid raft disruption or dispersal under both conditions. This supports the hypothesis that lipid raft disruptions precede the loss of epithelial barrier function.

We also used IL-10 knockout mice with mild colonic inflammation to further interrogate whether lipid raft disruption is associated with inflammatory microenvironments even in uninfamed/uninvolved intestinal regions. Conventionally housed IL-10 knockout mice spontaneously develop colitis and exhibit increased small intestinal permeability prior to the onset of inflammation (2, 29). Although our mice had no ileal inflammation, again we observed ileal raft disruption in the intestinal inflammatory microenvironment. An alternative raft isolation strategy (49) was used to exclude artifacts, since methods vary subtly via extraction of cholesterol or specific lipids/proteins (5, 49, 51). Detergent methods may induce membrane reorganization and raft coalescence whereas nondenaturing methods preserve membrane structure (45). The consistency with which we observed raft disruption (across mouse models and across raft isolation procedures) supports our conviction that raft disruption is a real feature of inflammatory microenvironments. This was supported in biopsies of UC patients with mild or quiescent inflammation, in which flotillin-1 distribution was broadened and alkaline phosphatase activity reduced or shifted relative to that in control biopsies from patients undergoing cancer colonoscopy screening. Although no control model is ideal given the complexities of human subjects, the use of tissue from cancer screening patients still offers a better comparison than tissue from patients with non-IBD intestinal disease.

Further exploration of a larger number of patient samples revealed the trend toward a more diffuse pattern of activity for the lipid raft marker enzyme alkaline phosphatase in gradient fractions from UC patients (n = 12), relative to control patient
fractions in which alkaline phosphatase activity peaked in a single fraction of 22.5 ± 0.7% sucrose (n = 5 patients). Because of the heterogeneity of our patient population and a relatively limited number of samples (<20; which necessitated pooling UC active, UC inactive, and UC active/uninvolved cases together), it was not possible to detect statistically significant variation by using our methods. Nor was it possible to meaningfully detect proteins of interest such as flotillin-1, occludin, or ZO-1 in multiple samples per category (because of low protein concentrations even after acetone precipitation for fraction volume concentration). Nonetheless, we feel that trends from our collective data utilizing cells, mice, and humans suggest that subtle disruptions in lipid rafts occur early on in an environment primed for inflammation and/or barrier disruption.

In the context both of human ulcerative colitis and DSS murine colitis, it is particularly intriguing to speculate why lipid rafts might be disrupted in ileal tissue, which is classically considered to be uninvolved in the inflammatory process. Whether this is inconsequential or reflects a propensity to later develop inflammation/barrier disruption cannot be inferred from our study. However, it is interesting to note that ileal involvement in human ulcerative colitis (distinct from that in Crohn’s disease) has been reported in 6-17% of patients, most commonly involving the terminal ileum in phenomena known as backwash ileitis or retrograde ileitis (12, 14). Therefore we feel that a full investigation of the cellular mechanisms of lipid raft disruption in inflammatory microenvironments (despite histological evidence of “normality” or a functional epithelial barrier) deserves consideration for their potential contribution to later inflammation. It would also be particularly interesting to determine whether lipid raft disruption accompanies or precedes the intestinal leakiness that is characteristic of healthy next-degree relatives of IBD patients (15, 17).

Finally, we asked whether key TJ proteins were displaced from rafts in uninvolved regions (considering both histologically normal and barrier-intact properties) within inflammatory microenvironments. However, we did not find consistent evidence of this in cells or tissues. Reduced occludin expression was more commonly observed in inflammatory microenvironments, although it is possible that other TJ proteins (e.g., nonraft-enriched claudins) could compensate for its loss in the epithelial barrier. IFN-γ has been shown to decrease occludin expression, possibly via phosphorylation of 3-kinase and NF-κB cross talk (3). Although it was surprising to note that the anti-inflammatory cytokine IL-10 also reduced the total protein expression of occludin in Caco-2 cells, it has previously been shown that IL-10 is a significant soluble factor released from lamina propria lymphocytes that is capable of reducing the barrier function of co-cultured intestinal epithelial cells (46).

Taken together, these findings illustrate the fact that the relationship between TJ protein loss or gain and inflammation is complex. In fact, enhanced claudin expression has been associated with IBD and colitis-associated carcinoma (37, 56). It is likely that any alterations in TJ protein expression disturb adhesion signaling and barrier function. Since none of our models was overtly “inflamed,” it may be that any extra-raft translocation of TJ proteins happens later on. Still, associations between TJ translocation out of rafts and loss of barrier function remain controversial, even in studies using similar cytokine combinations (6, 22). ZO-1 and occludin displacement outside rafts has been linked to profound barrier loss after Clostridium difficile intoxication (43). It will be interesting to test whether spatial reorganization of TJ proteins within epithelial membranes is temporally regulated during IBD inflammatory progression.

The possibility that TJ protein displacement from rafts might impair protein-protein interactions and contribute to dysregulated cell-cell adhesion is an interesting one. Dietary modifications that alter TJs have already been described (40), and it is intriguing to speculate that modifications that modulate membrane lipid content could regulate the onset or severity of inflammation via this mechanism. Polysaturated fatty acids (n-3 PUFA) enhance intestinal epithelial barrier function (57) and reportedly relocalize occludin and ZO-1 to lipid raft fractions (23). In vivo, n-3 PUFA supplementation has been shown to attenuate inflammation or promote remission in both murine (8, 32) and human (55) IBD.

In conclusion, our study has shown for the first time (to our knowledge) that disruption of intestinal epithelial lipid rafts is a feature of early-inflammatory or primed inflammatory conditions in cells, in mouse models of colitis, and in quiescent human IBD even in the absence of visible inflammation or significant epithelial barrier disruption. Evidence of lipid raft disruption even in the absence of overt damage to the epithelial barrier suggests that this may constitute an early signal in the inflammatory cascade that culminates in IBD.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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The molecular aspects of tight junctions

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1. Abstract

Tight junctions (TJs) are multi-protein complexes whose principal function is to mediate cell-cell adhesion between epithelial or endothelial cells. While once thought to participate solely as passive effectors of adhesion, it is increasingly being recognised that TJs are dynamic structures which regulate many aspects of cellular function and physiology. Accordingly, dysregulation of TJ-based adhesion or signalling is emerging as an intriguing contributor to several pathophysiology including cancer. This review will attempt to summarise the current state of knowledge about molecular aspects which regulate, and are regulated by, TJs. The first section will outline selected physiological processes known to influence TJ structure or function, under the headings of cell adhesion/polarity, cell-matrix signalling, ion transport, hormone effects, pro-inflammatory cytokines and hypoxia. The second section will describe selected functional behaviours within the pathophysiology of cancer which TJs have been demonstrated to influence, encompassing cell proliferation and apoptosis, migration and invasion, cell fate and differentiation, metastasis across the blood brain barrier and finally angiogenesis. Collectively, these sections illustrate that a wealth of mechanistic information can be gained from interrogating the contribution of TJs to normal physiology. In turn they highlight how TJ-based disturbances can promote some of the functional behaviours associated with cancer, and thereby offer insight into new TJ-based targets that may offer pharmacological promise in halting tumour progression.
2. Introduction

Externally, epithelial cells of the skin form a selective physical barrier between an organism and environmental insults including allergens and chemicals. Internally, the epithelial cells lining most visceral organs in conjunction with endothelial cells lining the vasculature also function as barriers to prevent absorption of pathogens and harmful substances from their external surfaces.

Tight junctions (TJs), adhesion complexes which connect the lateral membranes of adjacent epithelial or endothelial cells close to their external surfaces, are responsible for sealing the intracellular space and thereby creating separate apical and basolateral compartments \[1, 2\]. This intracellular TJ seal performs several crucial functions. Firstly, TJ proteins constitute a molecular barrier, which controls paracellular permeability and transport of ions, solutes and even cells. Secondly, homo- and hetero-typic binding of TJ proteins between neighboring cells aids in the establishment and maintenance of cell polarity by effectively linking polarity complexes with the underlying cytoskeletal structure of individual cells. Finally, TJ proteins can facilitate transmission of signals culminating in processes such as cell differentiation, growth, migration and invasion.

The molecular components of tight junctions can be broadly split into 3 main groups reviewed in detail in \[3\]: (i) integral transmembrane proteins including occludin, claudins, junctional adhesion molecules (JAMs), crumbs; (ii) peripheral or plaque adaptor proteins which generally contain PDZ domains that facilitate protein-protein interactions, such as the Zona Occludens (ZO) family, Par3, Par6, Arafad; (iii) associated regulatory/signaling proteins including cingulin and Rho-GTPases.

Deficits in tight junction function which lead to increased paracellular permeability have been linked to several pathologies including blistering skin diseases \[4\] and inflammatory bowel diseases (IBD) such as ulcerative colitis \[5\] and Crohn’s disease \[6\] (reviewed in detail in \[7\]). In addition, patients with IBD have a significantly higher risk of developing colitis-associated cancer, suggesting that efficient TJ barrier function may play a crucial role in preventing cancer development. Indeed, a wealth of evidence has recently associated alterations in several TJ proteins with many solid tumours including breast, lung colorectal, and gastric \[8\].

Common putative mechanisms of TJ dysregulation in cancer include aberrant microRNA regulation of gene expression (Eg. JAM-A in breast cancer \[9\]), aberrant methylation control (Eg. Claudin6 in breast cancer \[10\]) and protein mislocalisation (Eg. ZO-1 in pancreatic cancer \[11\]). Furthermore, common dysregulation patterns are evident across multiple cancer types, such as widespread down-regulation of claudin 1 and loss of ZO protein expression and localization\[8\].
Intriguingly, genetic classifications of human breast cancer subtypes describe a highly-aggressive subtype, the claudin-low subtype, which is characterized by reduced expression of claudins 3, 4 and 7 and accompanied by increased expression of proteins involved in epithelial to mesenchymal transition (EMT) [11]. Taken together, this wealth of data suggests that alterations in TJ functions may in fact play a causal role in cancer. The easiest interpretations are that simple downregulation of TJ proteins leading to barrier breakdown and concomitant loss of adhesion would disrupt cell polarity and promote cancer cell dissemination; or that upregulation or mislocalisation of TJ proteins could initiate aberrant signalling cascades that culminate in abnormal differentiation, proliferation, migration, and invasion. However, given the multifunctional roles of TJ proteins in several biological processes, it remains unlikely that a simple paradigm of either TJ protein loss or TJ protein gain will explain the complexities of different cancers.

In this review, we will firstly outline the processes that influence tight junction structure and function at a physiological level; then focus on emerging data describing mechanisms whereby alterations in TJ proteins may facilitate processes critical for tumor formation and progression. Given the huge expansion in the TJ literature over the last number of years, it has not been possible to give an exhaustive overview of all the relevant literature. We therefore chose to focus on some selected aspects, and apologise to those authors whose work we did not have space to include.

3. Physiological Processes Which Involve Tight Junction Proteins

The strategic location of TJs at the apical-most interface of the lateral intercellular membrane of polarized epithelia or endothelia facilitates their dynamic regulation by both extracellular and intracellular factors during a variety of physiological processes. Some of these will be summarized in the upcoming paragraphs.

3.1 Establishment of Cell Adhesion and Polarity

Tight junction proteins are critically important for the establishment of cellular adhesion and cell polarity through interactions with polarity complex proteins and RhoGTPases. For example, TJ barrier function is strongly regulated through homotypic binding of claudin proteins on adjacent cells, which control permeability through the formation of aqueous pores [12]. However, formation of apico-basal polarity requires the coordinate spatial regulation of the Par, Crumbs, and Scribble core polarity complexes.

Nascent cell adhesions are enriched in the TJ proteins JAM-A and ZO1, and also the
adherens junction protein E-cadherin (which activates Rac1 and suppresses RhoA to facilitate junction formation [13]). Upon initiation of cell polarity, Par3 is transported to the apical cortex [14] where its association with TJ proteins such as JAM-A [15] and PTEN [16] facilitates Par3-TIAM1 interactions which mediate stabilization and maturation of junctions [17]. Subsequent binding of the small GTPase Cdc42 to Par6 provides the stimulus for recruitment of atypical PKC (aPKC) to the apical surface, which serves to maintain apical domain integrity [18] and to recruit the Crumbs complex to the apical cortex via an interaction with PALS1 [19]. The basolateral-associated Scribble complex is formed through co-localization of Scribble [20] and Dlg [21] proteins at the basolateral cortex; with recent data suggesting that Scribble association with ZO1 may be critical for TJ assembly [22]. This data indicate that the interplay between TJ and polarity proteins is crucial for both maturation of apical junctions and the formation of apico-basal polarity.

3.2 Cell-Matrix Signaling

TJ proteins can also influence cell-matrix interactions, transmitting signals to and from the microenvironment to control cell polarity and processes such as migration and invasion. For example, JAM-A-deficient neutrophils show impaired activation of the small GTPase Rap1A [23], which is known to promote β1-integrin activation in a Talin-dependent manner [24]. JAM-A knockdown or inhibition using blocking antibodies has also been shown to reduce Rap1-GTPase activity and to decrease cell migration in colonic and breast epithelial cells [25, 26].

Correct cell-matrix interactions are also critically important for developmental processes involving the generation of multi-dimensional glandular structures such as acini or organoids. For example, activity of the extracellular matrix (ECM)-degrading protein, matrix metalloproteinase MT1-MMP, has been shown to be crucial for normal branching during mammary gland development [27]. Interestingly, the TJ protein ZO1 has recently been shown to regulate MT1-MMP expression in breast cell lines, suggesting that TJs may participate in modulating cell-matrix interactions during normal morphogenesis [28, 29].

Bidirectionality in the signalling cascades between cell-cell and cell-matrix complexes is also evident, as typified by functional interactions between the cell-matrix protein CD44 and TJs. CD44 binds ECM components such as hyaluronic acid, collagen, fibronectin, laminin, and chondroitin sulfate [30]. Recent studies have demonstrated that CD44 can regulate TJ assembly and barrier function in keratinocyte epithelial cells [31]. Specifically, CD44 knockout mice exhibited alterations in expression and/or localization of TJ proteins including Claudins-1 and -4, ZO1, and Par3; and a reduction in Rac1 activity culminating in a loss of cell polarity and
decreased epidermal barrier function.

3.3 Alterations in Ion Transport

A number of epithelial ion channels have emerged as important regulators of TJ function, of which the sodium potassium ATPase (Na⁺ K⁺ ATPase) is the best studied. This heterodimeric protein exports three sodium ions and imports two of potassium against their concentration gradients in an ATP-dependent reaction to maintain transmembrane ion concentrations [32-35]. This serves to maintain transmembrane potential, driving multiple transport mechanisms and controlling cell volume and osmolality.

A functional Na⁺ K⁺ ATPase plays important roles in the assembly of TJs, establishment of cell polarity and regulation of paracellular permeability. Studies in various epithelial cell types by Rajasekeran et al. [36, 37] [38] have shown reversible inhibition of TJ formation upon inhibition of Na⁺ K⁺ ATPase, either by K⁺ depletion or treatment with the inhibitor ouabain, in a sodium-dependent fashion. In addition, expression of Na⁺ K⁺ ATPase subunits and E-cadherin, in addition to adequate Na⁺ K⁺ ATPase pump function, have been shown to be necessary for TJ formation and normal epithelial polarization [36, 37]. Accordingly, it has been hypothesised that Na⁺ K⁺ ATPase and E-cadherin function synergistically in assembling TJs [38].

Interestingly, while treatment with high concentrations of ouabain that inhibit Na⁺ K⁺ ATPase pump function increase permeability and decrease transepithelial resistance [36, 39], treatment with nanomolar ouabain concentrations that do not affect pump function actually decrease TJ permeability to both ions and non-ionic molecules [40]. The latter has been attributed in part to alterations in the expression of claudins -1, -2 and -4 [40]. It is intriguing to speculate that such profoundly opposing effects on TJ function may in fact be subject to physiological regulation by endogenous hormone-like molecules in addition to exogenous drugs, with reports that an endogenous form of ouabain is synthesized and stored in the mammalian adrenal cortex and hypothalamus [41].

Other ion channels which have been implicated in regulatory control of TJ functions include the Na⁺/glucose co-transporter SGLT-1. Glucose uptake by apically-expressed SGLT-1 in the intestinal brush border has been shown to induce a drop in transepithelial electrical resistance and to increase in the paracellular uptake of small nutrients in vitro [42] and in vivo in both rats [43] and humans [44]. This has been associated with TJ strand disruption [45], dissociation of ZO1 from tight junctions [46] and phosphorylation of myosin regulatory light chain at the epithelial perijunctional ring [42].
Energy-dependent ion channels are not the only ones to have been functionally linked to TJs, with the passive transport chloride transporter CIC-2 also known to localise at TJs in intestinal epithelia. Activation of this channel reportedly stimulates an increase in transepithelial electrical resistance and a concomitant reduction in paracellular permeability [47]. Furthermore, expression of the chloride channel CFTR has been shown to increase transepithelial resistance [48]; while CLIC-4 co-localises with ZO1 in apical regions of epithelial cells, suggesting a possible but unproven role in regulation of TJs [49]. Finally, the transmembrane water channels termed aquaporins are also thought to regulate TJs, with aquaporin-5 in particular being shown to modulate epithelial paracellular permeability [42, 50].

3.4 *Hormone Effects*

Several hormones from the steroid receptor family and otherwise have been shown to regulate TJs, consistent with the physiological need to actively modulate tissue permeability or other important functions of TJs at different stages of development or hormonal cycles.

Among the most prominent, estrogen has been demonstrated to profoundly affect the TJs of sex hormone-sensitive epithelia ranging from reproductive tissue to the intestinal tract. In cervico-vaginal epithelium, oestrogen can reportedly decrease the resistance of both epithelial TJs and the lateral intercellular space via matrix metalloproteinase 7-induced modulation of occludin, with the net effect of increasing epithelial permeability [51, 52].

Oestrogen receptor-β (ERβ) is expressed in intestinal epithelial cells, where it appears to regulate paracellular permeability in a manner not strictly dependent on the oestrous cycle. In fact both male and female rats that under-express ERβ exhibit greater epithelial permeability and susceptibility to barrier-disruptive injury than their wild type female counterparts [53, 54]. Female rats under-expressing ERβ also show ultrastructural evidence of altered TJ and desmosomal morphology [53].

Hormonal regulation of mammary epithelial permeability during pregnancy and lactation occurs via not just the complex effects of oestrogen, but rather its interplay with other hormones such as progesterone, glucocorticoids, prolactin and serotonin (5-HT). During pregnancy the mammary gland reaches the expanded alveolar stage of development, however milk synthesis cannot begin until after parturition in conjunction with prolactin and glucocorticoid secretion which dynamically regulate TJ opening to facilitate the delivery of milk proteins during breastfeeding. [55-57].

The neurotransmitter serotonin (5-HT) also appears to regulate epithelial homeostasis in several organ systems including the mammary gland, where it is locally synthesized [58]. 5-HT
regulates the lactation to involution switch, and exhibits biphasic effects on tight junctions in vitro; increasing transepithelial resistance at low concentrations and early time points via protein kinase A while disrupting TJs via p38 MAP kinase signalling following sustained exposure to higher concentrations [59, 60].

3.5 Pro-Inflammatory Cytokines

Prototypic pro-inflammatory cytokines including interleukins-1, -6, -17, -18 (IL-1, -6, -17, -18), tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) are secreted from multiple cellular sources under physiological and pathophysiological circumstances. Among their pleiotropic effects include profound remodelling of TJs, which often induces endothelial or epithelial barrier disruption and perpetuates inflammation (for a recent review see [61]). The near-ubiquitous expression of cytokine receptors has fuelled reports of cytokine-induced TJ disruption in most epithelial and endothelial barriers, yet despite functional overlap between different cytokines there is no unifying paradigm of specific alterations which are essential for barrier dysfunction.

IL-1 has been reported to induce a variable combination of reduced transepithelial resistance or increased paracellular permeability in conjunction with occludin and ZO-1 degradation/ redistribution in epithelial cells from the intestine [62], thyroid [63] and cornea [64] in addition to models of the blood-brain barrier [65], blood-retinal barrier [66] and blood-testis barrier [67]. The barrier-disruptive mechanisms associated with IL-1 exposure in both intestinal [62, 68, 69] and corneal [64] epithelial cells as well as an in vitro model of the blood-brain barrier [70] have been ascribed to canonical NF-κB signalling via upstream activators such as MEKK and downstream effectors including myosin light chain kinase.

In addition to phenocopying several noted effects of IL-1 on barrier function and occludin/ZO-1 distribution, TNF-α has been observed to reduce the structural complexity [71] of claudin-containing TJ strands [72]. Also in common with IL-1 signalling mechanisms, TNF-dependent reductions in barrier function have been linked to activation of NF-κB in retinal endothelial cells [73] and corneal epithelial cells [74]. Barrier disruption downstream of TNF-α signalling in intestinal epithelial models has alternately been proposed to reflect expression enhancement of specific micro-RNAs targeting occludin for degradation [75] or enhanced removal of occludin from tight junctions via caveolar-mediated endocytosis [76].

Observations of IFN-γ-induced epithelial [77-79] or microvasculature endothelial [80] barrier disruption in conjunction with degradation/mislocalization of ZO/occludin proteins might seem to mirror the cellular mechanisms discussed above in response to IL-1 or TNF-α exposure.
However several lines of evidence suggest not only overlapping mechanisms but also unique ones whereby IFN-γ disrupts barrier function. One contends that the barrier-disruptive effects of IFN-γ in intestinal epithelia involve PI3-kinase/NFκB cross-talk [81]; another that macropinocytotic internalisation of occludin is responsible for induced deficits [82], while yet another possibility is that IFN-induced protease activation cleaves supporting TJ proteins such as claudin-2 [83]. Reported alterations in the lipid composition of membrane microdomains following IFN/TNF co-treatment [84] also offer a novel explanation for putative sub-membranous displacement of occludin and ZO-1 from tight junctions. Accordingly, although synergism between IFN-γ and TNF-α has been reported in many instances [85-87], it is interesting to note that this can be dissociated from the pro-apoptotic effects of some pro-inflammatory cytokines [87].

3.6 Hypoxia

The adaptive response to reduced oxygen tension, termed hypoxia, also plays an important role in influencing TJ structure and function in physiological and pathophysiological settings. Physiological differences in vascular perfusion between tissues dictate that some body compartments exist in normoxic states (e.g. lung alveoli) while others are relatively hypoxic (e.g. colon). In pathophysiological settings, hypoxia reportedly activates Notch signalling [88]; which in turn has been implicated in reducing gene expression of TJ components such as occludin and ZO-1 during EMT in airway epithelial cells [89]. While this implies that Notch activation secondary to hypoxia exerts a negative effect on lung epithelial barrier function, it is interesting to note that inactivation of Notch signalling may have a similar net effect in intestinal epithelia [90]. Whether this reflects innate differences in sensitivity to hypoxic signalling in tissues with disparate basal oxygen tensions, or merely illustrates the importance of carefully balancing Notch activity levels for barrier function homeostasis in any epithelial tissue is not yet clear.

What is clear is that regulation of epithelial barrier function by hypoxic signalling is complex and multi-factorial. Temporal activation of the transcription factors Slug and Snail during hypoxia [91] can also trigger junctional disassembly via repression of occludin, ZO-1 and claudin-1 expression [92-94]. Similarly, reduced expression of occludin and claudin-1 have been demonstrated both in vitro and in vivo in renal epithelial cells deficient in the tumour suppressor gene von Hippel-Lindau (VHL) [95]. Consequently, VHL inactivation has been associated with loss of barrier function and structural disorder of the renal epithelial phenotype [96]. Since a major function of the VHL protein product is to promote proteasomal degradation of hypoxia inducible factors (HIFs) [97], much interest has focussed on the potential role of HIFs in regulating TJs in various tissues. Recent evidence has suggested that HIF-1α antagonism can
temper occludin/ZO-1 redistribution and the associated defects in intestinal epithelial barrier function induced by pro-inflammatory cytokines [98]. A similar regulatory role has been noted in endothelial tight junctions, with loss of HIF-1α promoting TJ re-sealing in brain microvascular endothelial sheets compromised by prior exposure to either high glucose levels [99] or hypoxia-reoxygenation injury [100]. Taken together with the links between enhanced HIF activity and tumour progression or metastasis [101], the importance of hypoxia as an upstream regulator of tight junctions and barrier function cannot be underestimated.

As described above, TJ proteins are important for the maintenance of cell polarity and for hormonal and cytokine regulation of cellular homeostasis among a myriad of associated functions. Disruptions in cell polarity and tissue architecture are hallmarks of de-differentiation and early features of malignancy [102]. In addition, several TJ-associated proteins have recently been shown to be targeted by oncoproteins such as ERBB2 [103] and MYC [104]) to facilitate malignant transformation. Furthermore, TJ proteins including Scribble [105]and ZO2 [106] have been shown to be targeted by oncogenic viruses such as the human papilloma virus. Collectively, these studies provide strong evidence that TJ proteins may indeed act as key regulators of cancer initiation and progression. This will next be addressed.

4. Pathophysiological Processes in Cancer Influenced by Tight Junctions

4.1 Regulation of Cell Proliferation and Apoptosis

Tumour formation requires the acquisition of alterations that facilitate sustained proliferative capacity, whilst resisting cellular senescence and apoptotic cell death [107]. Several studies have described how alterations in several TJ-associated proteins may upset the delicate balance of growth and death signalling to result in malignant transformation. As noted earlier, however, the complex and tissue-specific regulation of TJ function in various endothelial and epithelial cells makes it unlikely that a single paradigm of simple expressional upregulation or downregulation will emerge to explain the many functional events associated with tumour initiation and progression.

Regardless, members of the largest family of integral membrane TJ proteins, the claudins, are frequently dysregulated in many cancers and appear to have a central role in determining cell fate [108]. With respect to tumour initiation, Claudin-6 downregulation has been shown to result in increased resistance to apoptosis in vitro [10]. Claudin-1 expression, though increased in senescent cells [109], has been reported to be decreased throughout several tumour types [8]. Similarly downregulation of occludin has been correlated with dedifferentiation and
progression of several cancers including endometrial [110] and lung [111]. These effects may be due to occludin-mediated regulation of apoptosis, as occludin loss results in decreased expression of pro-apoptotic proteins including Bax [112].

Alterations in proteins of the junctional adhesion molecule (JAM) family have also been shown in breast and renal cancers [25, 113] and melanoma [114], where many have demonstrated prognostic value in determining levels of JAM-A expression in patient cohorts. Indeed aberrant expression of microRNA mir145 may be mechanistically responsible for observed overexpression of JAM-A in breast cancer patient tissues which correlate strongly with poor survival outcomes [9, 115]. Although generally accepted as primarily functioning in adhesive and barrier roles at the TJ, compelling data have recently emerged regarding a role for JAM-A in both apoptosis and proliferation control. Colonic epithelial cells of JAM-A-deficient mice show enhanced crypt proliferation as measured by Ki67 staining [116]. Specifically, JAM-A appears to control cell proliferation through inhibition of Akt-dependent β-catenin activation [117]; with Akt inhibition reversing crypt proliferation in JAM-A-deficient mice. Somewhat conversely in the context of cancer, JAM-A deficient mice display significantly reduced tumor growth in a pancreatic tumor model, likely due to decreased angiogenesis and increased immune responses [118]. Furthermore, in a breast cancer mouse model, JAM-A deficient mice show significantly decreased tumour growth; with tumour cells displaying increased rates of apoptosis in vivo and in vitro [119]. Together these studies suggest that, in contrast to loss of TJ proteins such as claudins and occludin, upregulation of JAM-A may in fact facilitate increased tumor growth and survival by promoting signalling events which protect cells from apoptosis.

As mentioned, JAM-A associates with the peripheral TJ protein Par3 during junctional maturation and establishment of cell polarity [120]. In a mouse model of mammary morphogenesis, Par3 depletion in mammary progenitor cells disrupted mammary development, resulting in ductal hyperplasia. Re-expression of full length Par3 (but not truncated Par3) rescued this defect, demonstrating that Par3/aPKC interaction is essential for normal breast morphogenesis [121]. The interaction of another TJ-associated protein and Par polarity complex member, Par 6, with aPKC has also been shown to be required for ErbB2 oncogene-driven evasion of apoptosis and disruption of breast cellular morphogenesis in vitro [103].

Interestingly, association of ZO-1 with the transcription factor ZONAB can directly promote expression of the ErbB2 oncogene [122]. Furthermore, ZONAB is a critical determinant of cell cycle progress through effects on cyclin D1 and cdk4 [123]. Similarly ZO2 can control cell proliferation through sequestration of transcription factors such as Jun and Fos at the TJ in a density-dependent manner [124]. Finally, interactions between ZO1 with the polarity complex
member Scribble play an important role in normal regulation of cell adhesion [22]. Interestingly, correct localization and expression of Scribble mediates pro-apoptotic signalling critical for both normal mammary gland morphogenesis and resisting MYC-induced transformation [104].

In summary, data suggests that TJ proteins may be critical determinants of cancer initiation through effects on oncogene expression and imbalances in cell proliferative and apoptotic signaling.

4.2 Migration and Invasion

Although uncontrolled growth is a fundamental requirement during transformation, cancer cells must acquire both migratory and invasive capabilities in order to successfully disseminate from a primary tumour before seeding metastases at distant sites. Generally, cell migration consists of three main steps: the activation of Rho GTPases extend cell protrusions (through assembly of focal contacts with extracellular matrix proteins), the cell is dragged forward (through myosin II-mediated cell contraction), and finally cell adhesions are disassembled at the rear of the cell. This cyclical process (similar to regulation of apico-basal polarity and establishment of cell adhesion) requires crosstalk between junctional proteins, core polarity regulators [125, 126], and Rho family GTPases [127, 128]. Malignant cells can hijack these pro-migratory pathways and several TJ associated proteins have been implicated as having a causal role in cancer progression.

Loss of claudin-7 expression has been correlated with increased migration and invasion in lung [129], colorectal [130] and oesophageal cancer [131]. Specifically, claudin-7 loss or mislocalisation in oesophageal cancer can lead to decreased E-cadherin expression and increased 3-dimensional invasion in vitro [131]. Furthermore, re-expression of claudin-7 in claudin-7 deficient lung cancer cells resulted in decreased hepatocyte growth factor-mediated in vitro migration and invasion, and decreased in vivo tumour growth via regulation of ERK/MAPK signalling [129]. Several other claudins have been implicated in regulating invasion through effects on matrix degrading enzymes from the matrix metalloproteinase (MMP) family. Claudin-1 expression in liver cancer cells promotes increased MMP2 activity and migration and invasion through activation of a c-Abl-PKCdelta signaling pathway [132]. Conversely, claudin-6 loss has been demonstrated to increase MMP activity and promote invasion of breast cancer cells [10].

JAMs also have established roles in promoting normal leukocyte [133] and neutrophil migration [23], with JAM-A loss in endothelial cells functioning to decrease motility [134]. With respect to cancer, the majority of studies suggest that JAM proteins signal to increase cancer cell migration and invasion. JAM-A overexpression is associated with increased breast cancer
metastasis [115]; potentially due to downstream regulation of the migratory protein β1-integrin through AF-6 and Rap1 GTPase adaptor proteins [25, 26]. Furthermore, JAM-C is required for melanoma cell transendothelial migration \textit{in vitro} [135]; with its increased expression linked to melanoma invasion and metastasis \textit{in vivo} [136].

JAM proteins interact with several TJ adaptor proteins including AF6 and ZO proteins [1]. Fusion of AF6 and MLL represents the most common alteration in mixed lineage leukemia (MLL), where the Ras association-1 domain of AF6 likely activates the oncogenic potential of the MLL-AF6 protein [137]. Recently, loss of AF6 in breast cancer has also been linked with poor prognosis [138]. Further work has demonstrated that AF6 loss dramatically increased heregulin-induced \textit{in vitro} migration and invasion through activation of RAS/MAPK and Src kinase pathways; as well as significantly increased tumour growth and metastasis in an SKBR3 orthotopic mouse model [139].

Interestingly, ZO1 has been shown to regulate the expression of the matrix metalloproteinase MT1-MMP, with knockdown of ZO1 in breast cancer cell lines reducing MT1-MMP expression and 3-dimensional \textit{in vitro} invasion [28]. Recently, the TGF-β/Smad pathway (known to target the Par polarity complex [140]) was demonstrated to induce breast cancer cell invasion through up-regulation of MMPs -2 and -9, reinforcing a potential link between matrix degradation and TJ-associated proteins [141].

Furthermore, interactions between the Par complex members Par6 and aPKC leads to Rac GTPase activation in non-small cell lung cancer (NSCLC) cells, which drives anchorage-independent growth and invasion through activation of MMP10 [142]. The evidence for an involvement of Par3 in cancer cell migration has also been strengthened by studies demonstrating that Par-3 engages in the spatial regulation of Rac activity. Par3 directly interacts with Tiam1, a Rac1-specific guanine nucleotide exchange factor, to form a complex with aPKC-PAR-6-Cdc42, leading to Rac1 activation [17]. Recently, Par3 has been suggested to also be important in regulating squamous cell carcinoma collective cell migration. Recruitment of Par3 by DDR1 reduced actinomyosin contractile activity at cell-cell contacts and antagonized ROCK activity to Rac activation, thus keeping migrating cells clustered together and promoting more efficient collective migration [143].

Finally loss or mislocalisation of the ZO1 interacting protein, Scribble, increases migration and invasion of breast cancer cell lines [104, 144], and cooperation of Scribble with the Ras oncogene increases MEK-ERK-dependent matrix invasion in a 3D breast acinar morphogenesis model [145].

Together, the above studies underline the importance of TJ proteins in mediating pro-
migratory and pro-invasive signals and also suggest that targeting these proteins in cancer may be of therapeutic value.

4.3 Cell Fate and Differentiation

Recent work has provided evidence that several TJ proteins may regulate cell fate and differentiation during normal development [146, 147]. Expression levels of Claudin-4, ZO1 and ZO2 regulate murine stem cell commitment to hematopoietic or endothelial cell lineages [148]. In addition, JAM proteins have been shown to be required for maintenance of hematopoietic stem cells in bone marrow [149], spermatid differentiation [150] and dendritic cell differentiation [151]. Furthermore, as mentioned above TJ proteins interact with polarity complexes such as the Scribble and Par complexes to influence cell fate through processes including EMT, which allows cancer cells to alter their cell morphology and acquire pro-invasive phenotypes that might facilitate their migration to optimally-supportive growth niches [140, 145, 152].

The claudin-low aggressive breast cancer subtype is characterized by near absence of luminal differentiation markers, and increased expression of EMT and cancer stem cell-like markers [11]. Indeed, gene expression signatures derived from normal human breast cells undergoing EMT in response to snail/slug activation or TGFβ treatment were recently shown to closely resemble those derived from claudin-low breast cancer tissues [153]. Poor prognosis claudin-low tumour cells could undergo EMT through changes in several Zeb1 transcription factor–regulated genes. Zeb1 expression, through its repression of junctional proteins, may therefore also have a causal role in cancer types including breast [154] and colorectal [155]. Downregulation of Mir200c in breast cancer cells prevents expression of Zeb1, and reduces cancer cell migration [156]. Furthermore, knockdown of Zeb1 in MDA-MB-231 breast cancer cells promotes EMT reversion whereby induced re-expression of the TJ proteins JAM-A, Occludin, Crumbs and PATJ partially re-establishes cell polarity and epithelial morphology, and significantly decreases cancer cell migration. Encouragingly, Zeb1 knockdown in a mouse model of metastatic colorectal cancer resulted in complete suppression of liver metastasis [155], suggesting that targeting Zeb1 may be a valuable therapeutic modality.

The TJ peripheral protein Par6 has been demonstrated to be required for TGFβ-induced EMT in breast epithelial cells [140]. Specifically, TGFβ-dependent phosphorylation of Par6 mediated recruitment of Smurf 1 (an E3 ubiquitin ligase) to promote degradation of RhoA and dissolution of the TJs, a crucial step in EMT [152]. In addition, TGFβ-Par6 signalling led to a loss of cell polarity and induced local invasion of MMECs in vitro and in vivo [140].

ZO1 and its associated transcription factor ZONAB have also been implicated in the
regulation of epithelial homeostasis and differentiation [123, 157, 158], through downstream regulation of cell cycle genes such as cyclin D1 and PCNA. Overexpression of ZONAB or knockdown of ZO-1 in mouse epithelial cells resulted in increased proliferation, and induced EMT-like morphological and protein expression changes that disrupted normal epithelial differentiation. This suggests that ZO1 loss, as seen in several cancers [159, 160], may phenocopy ZONAB over-expression in vitro thus altering cell differentiation through the induction of EMT.

Finally, several recent studies have suggested novel roles for TJ-associated proteins in controlling cellular homeostasis through regulation of spindle orientation and cell division. As mentioned above, transplantation of Par3-depleted stem cells into murine mammary fat pads resulted in disrupted ductal morphogenesis [121]. Interestingly, however, an expansion in the luminal progenitor cell population and reduction in myoepithelia cell population was evident in Par3-depleted mammary glands. This suggests a role for Par3 and its TJ binding partners in the regulation of progenitor differentiation and epithelial morphogenesis in vivo.

Recent studies in MDCK renal epithelial cells have shown that Par3 knockdown disrupts aPKC association with the apical cortex, and causes spindle misorientation leading to the appearance of multiple lumens in 3D cysts [161]. Similarly, depletion or inhibition of Par6B or aPKC induces misorientation of the mitotic spindle to drive formation of aberrant Caco-2 intestinal epithelial cysts, with cell survival and apical positioning dependent upon aPKC expression levels [162]. Together these results suggest that TJ-associated proteins may have a role in spindle orientation and cell differentiation in vivo, and that their alteration may facilitate tumour formation by affecting the spatial regulation of cell division.

4.4 Metastasis and the Blood-Brain Barrier

In addition to their regulatory roles in cell fate and differentiation, the functional integrity of TJ proteins also play an intrinsic part in preventing cancer metastasis. In order for metastasis to occur, invading cells must first detach from the primary tumour and invade into the bloodstream. At the site of metastasis, the tumour cells must extravasate. This is similar to leukocyte extravasation and consists of three steps; firstly loose attachment and rolling on the endothelial surface, secondly tight attachment to the endothelium, and thirdly diapedesis or transmigration through the endothelium, either by the transcellular or paracellular route. While leukocytes and tumour cells share many similarities during the first two steps, the third step of diapedesis differs in that tumour cell migration irreversibly alters endothelial morphology [163]. This in turn induces endothelial cell retraction and in some cases apoptosis, possibly due to loss of cell-cell contacts [164-166] via molecules including N-cadherin [167, 168]. The net effect of this destructive form
of transmigration is the formation of gaps in the endothelial barrier, which can allow permissive access of tumour cells to the circulation and facilitate the early steps of metastasis.

A unique and highly-specialised form of the endothelium that poses a significant barrier to metastasis is the blood brain barrier (BBB), a complex structure consisting of nonfenestrated brain microvascular endothelial cells (BMECs) held together by abundant TJs and adherens junctions [reviewed in [169, 170]]. Relative to other endothelial cells, BMECs exhibit higher transepithelial resistances and lower solute permeability, while TJs are structurally more complex and restrictive to diffusion of polar solutes via the paracellular pathway. The basement membrane is also thicker, and a layer of underlying astrocytes adds an extra regulatory component that restricts flow across the barrier. Collectively, the layers that compose the BBB represent a formidable challenge to the cancer cells which must breach it in order to form brain metastases. Since the brain lacks lymphatic drainage, brain metastasis occurs solely via the haematogenous route. It often carries a dire prognosis due to limited available treatment modalities, since the BBB is as impermeable to most drugs as it is to cells. Thus in recent years much interest has focused upon the regulation of BBB TJs, both to prevent cross-trafficking of tumour cells and also to understand mechanisms of selectively enhancing permeability to facilitate chemotherapeutic drug delivery. In particular, claudins have emerged as promising targets since losses in claudins-3 and -5 have been associated with increased leakiness of the BBB [171, 172].

With regard to specific types of cancers, melanoma displays the highest propensity of all primary tumours to metastasize to brain [173]. Melanoma cells have been shown in vitro to degrade brain endothelial TJs, resulting in decreased transepithelial resistance and decreased expression of claudin-5, ZO-1 and occludin [174]. While the mechanisms of such events remain incompletely understood, TJ protein degradation may be facilitated by the fact that melanoma cells express several proteases, including matrix metalloproteinases [175], urokinase type plasminogen activator [176], seprase [177] and serine proteinases [174].

Similarly, breast cancer displays a high propensity for brain metastasis, with a prevalence of approximately 30% at autopsy [178, 179]. Risk factors include young age, grade and stage, oestrogen receptor negativity and Her2/neu overexpression [180, 181]. While many soluble and cell-fixed factors are potentially involved in the transit of breast cancer cells across the BBB, one intriguing pathway that has recently emerged involves the chemokine stromal cell derived factor-1α (SDF-1α) and its receptor CXCR4. SDF-1α is secreted by several organs including the central nervous system, and SDF-1α treatment has been shown to increase the permeability of BMEC monolayers to breast tumour cell invasion by activating the PI-3K/AKT signalling pathway and causing endothelial cell retraction [182]. Interestingly CXCR4 is expressed on breast cancer cells
and is sensitive to upregulation by the oncogene Her2/Neu, which is associated with aggressive and highly-metastatic forms of breast cancer [183]. As with melanoma metastasis, however, it is clear that many other pathways could also govern transit of breast cancer cells across the BBB. Some of these mechanisms are likely to be convergent, for example the activity of degradative enzymes such as MMP-2 and -3 has been shown to be increased in in vivo breast cancer models [184, 185], while that of MMP-1 & -9 has been shown to be increased in in vitro settings of breast cancer [186]. Given the links between MMPs and TJ's alluded to earlier in this article, cross-regulation in the context of enhancing BBB permeability could therefore have serious implications for the development of brain metastases.

4.5 Angiogenesis

Metastasis is a complex and multi-step process which requires many forms of sophisticated functional adaptation in addition to the relatively simple requirement for mechanical movement of cells across biological barriers. One biological process which exerts a key influence on the ability of metastasized tumours (and indeed primary tumours) to survive is the generation of a vascular supply to nourish the growing tumour, termed tumour angiogenesis. Several TJ proteins have been implicated in both physiological and pathological angiogenesis. Junction adhesional molecules, and in particular JAM-A, are known to be important regulators of angiogenesis. JAM-A is expressed in the early vasculature of the developing mouse embryo [187], and is a vital component of basic fibroblast growth factor (bFGF)- induced angiogenesis. In the latter context it forms an inhibitory complex with αvβ3 integrin, which disassembles in response to bFGF signalling. JAM-A then facilitates MAP kinase activation, which in turn induces endothelial tube formation and angiogenesis [188, 189]. Accordingly, transient knockdown of JAM-A has been shown to prevent bFGF-induced endothelial cell migration in an ECM substrate-specific fashion [189]. Similarly, bFGF cannot induce angiogenesis in JAM-A deficient mice [190], and pancreatic islet cell carcinomas grown in JAM-A null mice have been shown to exhibit a small decrease in angiogenesis compared to JAM-A-expressing mice [191].

Other JAM proteins also appear to regulate angiogenesis in ways that could be relevant to tumour angiogenesis, or the pharmacological antagonism thereof. Soluble JAM-C levels have been shown to be increased in the serum of patients with rheumatoid arthritis, and treatment with exogenous JAM-C has the potential to induce angiogenesis in vitro [192]. Furthermore JAM-C blockade has been shown to reduce angiogenesis by 50% in a mouse model of hypoxia-induced retinopathy [193]. Others have reported that functional antagonism of JAM-C with a monoclonal antibody can inhibit angiogenesis both in vitro and in vivo [192, 194]. Conversely, overexpression
of JAM-B in a mouse model of Down's syndrome has been shown to inhibit the angiogenic response to vascular endothelial growth factor (VEGF) [195].

Other TJ proteins such as the claudins also play a complex role in angiogenesis. Claudin-5 has been shown to reduce endothelial cell motility via N-WASP and ROCK signalling cascades [196]. Claudin-4-expressing ovarian epithelial cells reportedly feature upregulation of several genes encoding pro-angiogenic cytokines, and can induce angiogenesis both in vitro and in vivo mouse models [197]. Claudins -1, -2 and -5 are expressed in normal murine retinal vessel development; while claudins -2 and -5 are overexpressed in vessels in an oxygen-induced retinopathy model [198].

Similarly expression of occludin can be altered by a number of angiogenic factors. Increased occludin expression has been linked with the secretion of angiopoetin-1 from pericytes [199], while decreased occludin expression in conjunction with increased paracellular permeability has been noted in retinal endothelial cells treated with vascular endothelial growth factor (VEGF) [200, 201].

Taken together, the above points illustrate a complex and dynamic relationship between TJ proteins and angiogenic cascades. We believe this shows much potential for interrogation to better understand not only the mechanisms of tumour angiogenesis, but also to drive forward the design of new TJ-based therapeutics aimed at interfering with this process.

5. Conclusion
This review has attempted to summarise the molecular aspects of TJs regarding their regulation by normal physiological processes and their contributions to pathophysiological behaviours characteristic of cancers. What has emerged is that TJs are intrinsic downstream components of a number of important cascades regulating physiological processes as diverse as polarity, ion transport and responsiveness to paracrine or endocrine factors. Perhaps more importantly, it has also illustrated that while TJs may act as upstream regulators of functional behaviours intrinsically associated with cancer, there is no universal paradigm whereby simple loss or gain of TJ proteins drives processes like cell proliferation, migration or angiogenesis. Instead this review suggests that complex spatial and temporal regulation of TJ signalling must be elucidated on an individual protein basis, but may bear fruit in the design of future drugs to target tumourigenic behaviour.
Ductal Barriers in Mammary Epithelium

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Abstract: The mammary ductal epithelium is a dynamic barrier which is actively regulated in both physiological and pathophysiological settings. Under physiologically normal conditions, breast tight and adherens junctions undergo dynamic changes in permeability in response to hormonal and other stimuli. In the pathophysiological setting of breast cancer, emerging information suggests an exciting role for constituent proteins of both tight and adherens junctions in driving or modifying mammary tumorigenesis. This review describes firstly the structure of the mammary ductal epithelial barrier and its role in normal mammary development, examining the cyclical changes in response to puberty, pregnancy, lactation and involution. It next examines the role of adherens and tight junction proteins in mammary tumorigenesis, specifically their participation in functional such as migration, invasion and metastasis. Finally, the review discusses the potential of adhesion proteins as both prognostic biomarkers and potential therapeutic targets in breast cancer.

Introduction

Breast cancer is the commonest malignancy among women worldwide, with over 1.3 million new cases diagnosed and over 450,000 deaths per annum (1). While the prognosis of breast cancer has improved significantly in recent years, its mortality remains significant. Despite much research into targeted treatment modalities, treatment remains focused on surgical excision, chemotherapy, radiotherapy and hormone therapy, with the anti-HER2 monoclonal antibody trastuzumab currently the only targeted therapy in routine clinical use. As many of these treatment modalities are associated with significant side effects, the quest continues to discover biomarkers identifying those likely to benefit
most from adjuvant treatment, and in additional novel targets for development of anti-
breast cancer agents with minimal systemic side-effects.

Proteins involved in tight and adherens junctions are obvious candidates for such
biomarkers and/or therapeutic targets for a number of reasons: firstly, cellular junctions
courage physical cell-cell associations that must be overcome to allow tumor cell
shedding and distal metastasis, and secondly, many of the proteins in tight and adherens
junctions are also involved in pro-proliferative and pro-migratory signaling cascades
relevant to cancer progression. As the majority of breast cancer mortality is due to
metastatic disease rather than the primary tumor, the development of strategies to prevent
such distal spread is particularly crucial.

This review aims to examine the role of ductal adhesion complexes and their proteins in
both the normal and diseased breast. We will firstly discuss their structure and physiology
under normal circumstances such as puberty, pregnancy and lactation. Next we will
examine their role in abnormal breast conditions, both inflammatory and neoplastic, but
will focus on breast cancer as the most significant and best studied pathological condition
of the breast. We will discuss the role of cell junctions and their proteins in such
neoplastic behaviours as dysregulated proliferation, migration, invasion and metastasis.
Finally, we will examine the potential use of junctional proteins both as breast cancer
prognostic biomarkers and as therapeutic targets.

Structure of Mammary Ductal Epithelium

The mammary gland is a modified apocrine sweat gland that consists of multiple
pyramidal lobes each subdivided into several lobules, which in turn consist of multiple
acini. These drain via a complex network of branching ducts, eventually conveying milk
to the nipple. These structures are supported within a network of fat and connective tissue
(2). The basic functional unit is the terminal duct-lobular unit (TDLU), consisting of a
lobule and its draining duct.

Microscopically, mammary ducts are lined by a single luminal layer of columnar or
cuboidal epithelial cells surrounded by a discontinuous layer of contractile myoepithelial
cells, in turn surrounded by basement membrane (3). Like other epithelial layers,
mammary ductal epithelial cells are joined at their cell-cell interfaces by junctional
complexes; which include tight junctions, adherens junctions and desmosomes.

The tight junction forms a continuous band around the cell at the apical-most surface,
effectively dividing it into apical and basolateral compartments and determining the
cellular barrier and paracellular transport (4). Tight junction proteins can be broadly
divided into integral transmembrane proteins such as occludin, claudins and junctional
adhesion molecules (JAMs); peripheral or plaque adaptor proteins such as the Zona
Occludens (ZO) proteins; and regulatory/signaling proteins such as cingulin and the Rho-
GTPases (5). Adherens junctions are located subjacent to tight junctions, forming a band
around the cell and attaching the actin cytoskeleton to the plasma membrane (6). Their
proteins include the armadillo proteins (β-catenin, plakoglobin); cytoskeletal adaptor proteins like α–catenin; and the cadherins (E-cadherin in normal epithelial cells, N-cadherin in mesenchymal cells) (6-8). Desmosomes appear as patches subjacent to adherens junctions. They anchor keratinous intermediate filaments to the plasma membrane, while their proteins include cadherins (desmogleins, desmocollins), armadillo proteins (plakoglobin, plakophilins) and desmoplakin (8). While desmosomes are of lesser importance in breast epithelium, many of their proteins are also constituents of adherens junctions and may be involved in breast pathologies.

As detailed below, several of the above structures are modulated dynamically in time with the normal reproductive and hormonal cycle, in addition to being altered in many pathological processes.

**Biology of Mammary Ductal Barriers**

More than most organ systems, the breast undergoes frequent changes in response to puberty, the menstrual cycle, pregnancy, lactation and menopause; and many of these are modulated via alterations in ductal junctional complexes or junctional proteins directly. Signaling via the canonical Wnt pathway involving the adherens junction protein and transcription factor β-catenin has been implicated in virtually every stage of this cycle (9). Wnt/β-catenin signaling is vital for the formation of the embryonic mammary placode (10) and β-catenin target genes are upregulated in embryonic ductal morphogenesis (11). There is indirect evidence of low level Wnt/β-catenin signaling occurring during mammary development at puberty, with enrichment of Wnt5a and Wnt7b mRNAs in terminal end buds and that of Wnt2 in mammary stroma (9, 12-14). During pregnancy, progesterone-induced changes such as increased ductal branching are modulated via β-catenin signaling (15).

Pregnancy is characterized by increased leakiness of the mammary ductal tight junctions in particular, with numerous animal studies showing extravasation of large molecules from the pregnant duct (16-18), although to a greater extent in alveolar than ductal epithelium. This is reflected in the loss of trans-epithelial electrical resistance in the mammary epithelium of pregnant animals (19) and the altered composition of milk prepartum, with higher concentrations of the interstitial ions sodium and chloride, as well as proteins (18). In addition, morphological alterations in tight junctions have been observed during pregnancy, with lower numbers of strands and less branching complexity exhibited (20, 21).

Mammary epithelial tight junctions are altered by several hormones including glucocorticoids (22), prolactin (23), serotonin (24) and progesterone, and it is the sharp fall in the latter at parturition that allows tight junction closure to provide a leak-proof duct and restore trans-epithelial resistance, thus facilitating lactation (25). Interestingly, neutrophils are able to diapedes through these intercellular junctions to reach the lumen if necessary, with complete reconstitution of tight junctions occurring afterwards (26).
The neurotransmitter serotonin (5-HT) is also locally synthesized in the breast (27), where it exerts biphasic effects on the mammary tight junction, promoting tight junction integrity at low concentrations via protein kinase A; while following sustained exposure to higher concentrations tight junctions are disrupted via p38 MAP kinase signaling, encouraging mammary involution (24, 28).

Pathobiology of Mammary Ductal Barriers – Inflammation

As in many other organ systems, inflammation affecting the breast (mastitis), which most commonly occurs in the lactating gland, causes increased permeability of the ductal epithelium. This is evidenced by increased milk sodium and chloride content, as well as loss of trans-epithelial electrical resistance (18, 29). While this may in part be due to direct epithelial injury, tight junction permeability is also increased as part of the inflammatory response, mediated by inflammatory cytokines such as tumor necrosis factor (TNF) (30), histamine (31) and interferon-γ (32), and represents an important adaptive response to allow access by immune cells.

Pathobiology of Mammary Ductal Barriers – Breast Cancer

While some of the roles of ductal barriers in normal breast physiology and benign conditions have been described above, it is the role of mammary epithelial junctions and their proteins in the pathophysiology of breast cancer that has attracted by far the most attention. We will discuss below the role of ductal barriers and their constituent proteins in the pathophysiology of breast cancer, with particular focus on core markers of neoplastic behaviour such as dedifferentiation, proliferation, migration, invasion and metastasis; before discussing the potential roles of junctional proteins as tumor biomarkers and drug targets.

Cell Polarity and Dedifferentiation

Tight junctions are vital to maintaining polarity of epithelial cells, discriminating as they do between their apical and basolateral aspects. Tight junctions assemble three complexes that maintain cell polarity: CRB, PAR and Scribble. The CRB complex is the most apically located, and includes the proteins CRB3, PALS1 and PATJ; it defines the apical region for targeting of cytosolic proteins in epithelial cells. The PAR complex consists of Par3, Par6, atypical PKC (aPKC) and Cdc42/Rac1. Par3 interacts with JAM-A (33) and PTEN (34), which in turn allows it to interact with TIAM-1, stabilising the junction (35). Further binding of Cdc42/Rac1 recruits aPKC to the apical surface, maintaining the integrity of the apical cellular region (36). The basolateral scribble complex consists of the proteins Scribble, lethal giant larvae homolog (LGL) and discs large homolog (DLG) (37, 38). Scribble has been shown to co-localise with both the tight junction protein ZO1
(39) and with E-cadherin and DLG at adherens junctions (40), suggesting its importance in the formation of both these junctions, in addition to targeting CRB3 to apical regions (41), thus maintaining both the apical and basolateral domains.

Loss of cell polarity is a crucial step in epithelial-mesenchymal transformation (EMT), the process whereby epithelial-derived cancers, including breast, progressively develop an invasive mesenchymal signature and phenotype (42). Many proteins involved in cell polarity are dysregulated in breast cancer. The transcription factor ZEB1, which is upregulated in some breast cancers and is implicated in EMT, inhibits the expression of CRB3 and PATJ (43). Overexpression of Par6 in breast epithelial cells induces increased proliferation while maintaining cell polarity (44), and activation of ErbB2 (the gene encoding HER2, overexpressed in some aggressive breast cancers) disrupts apical-basal polarity by associating with Par6 and aPKC (45). Scribble expression in breast cancer specimens has been shown to be quantitatively reduced (40), while it is redistributed from the membrane in several breast cancer cell lines (46). In addition, its loss in mammary epithelial cells results in abnormal morphogenesis both in vivo and in vitro and inhibits c-myc-induced apoptosis in vitro (46).

Tight junction proteins have also been implicated in breast cellular dedifferentiation, frequently one of the earliest histological indicators of malignancy. Occludin is downregulated in breast cancer, and its loss correlates with a less differentiated phenotype (47), as does that of its interacting protein ZO-1 (48). JAM-A plays an important role in regulating cell morphology by modulating Rap1 activity (49). Despite initial conflicts between reports of its expression correlation with the malignant breast phenotype (50, 51), the balance of evidence now favors a model whereby JAM-A overexpression in breast cancer associates with poor prognosis (52-54)(5).

The cadherin switch is an important precursor of EMT in breast cancer. It involves a progressive dedifferentiation, switching from expression primarily of epithelial markers such as E-cadherin and cytokeratins to mesenchymal markers such as N-cadherin, vimentin and fibronectin (55). This may be a normal component of processes such as wound healing and development of structures such as tubules (56), however it occurs in a dysregulated fashion in cancer. E-cadherin is underexpressed in a number of breast cancer cell lines including the highly-invasive triple negative MDA-MB 231, although E- and N-cadherin status is not fully predictive of invasiveness (57). Immunohistochemical staining of breast cancer specimens suggests that E-cadherin is lost in the majority of lobular, but not ductal, breast carcinoma (58-60), and occurs as early as the in situ stage in lobular carcinoma (61). Numerous mechanisms can result in the loss or downregulation of E-cadherin, of which loss of heterozygosity and mutations in the CDH1 gene which encodes it is commonly seen in lobular carcinoma (62, 63).

A number of transcription factors can co-ordinate the shift from E- to N-cadherin expression, largely controlled by the transcription suppressor Snail. The latter protein activates Zeb1, which binds to the E-cadherin promoter and blocks transcription of E-cadherin (64, 65). Further repressors that have been implicated include Slug, Twist, Zeb2, E12/47, Sip1 and ßEF1, in addition to hypermethylation of E-cadherin promoters
Furthermore, E-cadherin can be targeted for endocytosis and degradation secondary to the actions of tyrosine kinases such as Src, EGFR, FGF receptor, c-Met and IGF-1R (70); and can be directly degraded by matrix metalloproteinases (55).

The protein Twist, itself regulated by canonical Wnt1 signaling (71), downregulates E-cadherin expression, concurrently upregulates the expression of N-cadherin and can induce EMT. Twist overexpression is associated with dysregulated cell growth in murine tumors (71), and with multi drug resistance (72-74). N-cadherin promotes fibroblast growth factor (FGF) signaling by binding to and preventing the internalisation of its receptor, thus sustaining its pro-migratory and invasive effects via MAP kinase activation and matrix metalloproteinase 9 secretion (75).

**Dysregulation of Proliferation**

Cancer can essentially be considered a disease of dysregulated cell growth and proliferation. A crucial point is the loss of regulation of the cell cycle, resulting in uncontrolled cell division and abnormal growth (76). Loss of E-cadherin expression, in addition to facilitating cell detachment through its mechanical effect at adherens junctions, directly induces a number of pro-proliferative signaling pathways in breast cancer. E-cadherin interacts with the epidermal growth factor (EGF) receptor to modulate proliferation by suppressing pro-proliferative tyrosine kinase signaling (77). E-cadherin also binds and sequesters β-catenin in adherens junctions, thus its downregulation frees β-catenin to enter the nucleus and participate in pro-proliferative canonical Wnt signaling. Increased levels of cytosolic and nuclear β-catenin have been reported in up to 68% of breast cancers, and implies a poor prognosis (78-81). In addition, upregulation of other β-catenin signaling promoters such as Disheveled (82), LRP 6 (83) and a mutant LRP5 (84), as well as downregulation of the β-catenin inhibitor Wnt5a (85), are commonly seen in breast cancer. Several alternative pathways are known to increase β-catenin expression are activated in breast cancer, including the NF-κB pathway in ER-negative, HER2-positive tumors (86); Pin1 upregulation is proportional to increasing tumor grade, which is associated with increased β-catenin expression (87, 88); while loss of PTEN activates Akt and β-catenin resulting in increased proliferation in breast cells (89, 90).

Placental (P-) cadherin, a junctional protein usually expressed by mammary myoepithelial cells, is expressed in approx 30% of breast cancer cell lines (57) and up to 50% of invasive ductal carcinoma specimens (91). It is strongly expressed in the basal (classically ER, PR and HER2 triple negative) and HER2-overexpressing subtypes of breast cancer (92), and has been suggested as a routine biomarker for basal-like cancers (93, 94). It is associated with increased proliferation, a reduction in ER-α signaling, increased p53 and HER2 expression; and a poorer prognosis (92). The cadherin switch from E- to P-expression is described in embryonic development (94), with little evidence of its occurrence in breast carcinoma, where P-cadherin is more commonly co-expressed with E-cadherin (95, 96).
Migration and Invasion

A further hallmark of malignancy that facilitates tumor spread and thus survival is dysregulated migration and invasion. Broadly speaking, cell migration consists of five cyclical steps. It begins with the formation of protrusions known as pseudopodia at the leading edge, driven by actin polymerisation (97) controlled by the Rho GTPase Cdc42 and several downstream effectors (98). These form small transient adhesions to extracellular matrix near the leading edge, involving $\beta_1$, $\beta_2$ and $\alpha_2\beta_1$ integrins and other adaptor and signaling proteins. These recruit proteinases that then cleave extracellular matrix barriers (99, 100). The cell body translocates forwards driven by actin-myosin shortening (101). Release of adhesions at the rear of the cell and retraction of the rear complete the cycle (102). Normal epithelium and well-differentiated carcinomas tend to exhibit collective migration and invasion, whereby cell-cell interactions are retained and migration occurs in single sheets or strands (103). In contrast, inflammatory cells, mesenchymally-derived tumor cells and poorly-differentiated carcinomas with loss of strong cell-cell contacts tend to migrate individually (104).

The tight junctional protein JAM-A has a somewhat controversial role in breast cancer. While early evidence suggested that low JAM-A expression correlated with a less migratory and invasive breast cancer phenotype (50), an increasing body of evidence from our group and others would suggest otherwise. Specifically, JAM-A overexpression in breast cancer specimens correlates with poorer patient prognosis (51), and its expression has been shown to correlate with HER2 expression (105), ER negativity, higher grade, and aggressive luminal B, HER2 and basal subtypes of breast cancer. Knockdown or antagonism of JAM-A reduces migration and invasion in JAM-A expressing breast cancer cells (53), and JAM-A has been shown to regulate both $\beta_1$-integrin expression via Rap1 activity (49), and HER2 signaling and degradation (105). It is possible that seemingly contradictory effects are due to JAM-A underexpression impairing cell adhesion and polarity, thus favouring early malignant change; while its overexpression in the later stages might favour tumor progression via integrin-mediated pro-migratory signaling (5).

Several members of the claudin family have been reported to be up- or downregulated in a variety of cancers. Claudin 4 appears to regulate migration in both normal and malignant breast cells (106), and its expression in breast cancer correlates with higher grade and worse prognosis (107). Similarly, claudin 5 expression correlates with worse survival and its forced expression increases breast cancer cell motility (108). In contrast, claudins 6 (109) and 16 (110) have been shown to decrease breast cancer cell migration and invasion, and loss of claudin 6 conversely promotes breast cancer cell invasion (111). Both claudin 1 under-expression (112) and high expression (113) have been variously associated with an invasive phenotype. Interestingly, a new molecular subtype of breast cancer, “claudin low,” has been recently described, in which tumors are characterized by
low gene expression of claudin 3,4 and 7 in conjunction with an aggressive, basal-like phenotype (114).

Adherens junctional proteins are also strongly implicated in breast cancer migration and invasion. The cadherin switch to expression of mesenchymal cadherins most likely facilitates migration and invasion both by increasing tumor cells’ ability to detach from their normal surrounding epithelial cells, and by inducing inappropriate pro-motility signaling (115). Transfection of N-cadherin into E-cadherin-expressing breast cancer cells induces invasion and motility (57), while transfection of E-cadherin into highly invasive mesenchymal-like MDA-MB 231 cells reduces invasion and migration (116).

Metastasis

Migration and invasion are particularly important for the systemic spread of tumors, acting as a key early step in the multi-step cascade known as metastasis. Accordingly, cell junctions and their proteins have been reported to play an intrinsic role in preventing breast cancer metastasis. The primary event in metastasis involves detachment of cells from the primary tumor and invasion into the bloodstream, followed by extravasation at the site of metastasis. This is somewhat similar to extravasation of leukocytes in the immune response and consists primarily of three steps; firstly loose attachment and rolling on the endothelial surface, secondly tighter attachment of the tumor cells to the endothelium, and thirdly diapedesis or transmigration through the endothelium. The latter can occur either by the transcellular or paracellular route. While the loose attachment, rolling and tighter attachment steps in tumor cells are similar to leukocytes, diapedesis of tumor cells differs from leukocytes in that it permanently alters endothelial morphology (117), resulting in retraction of endothelial cells and in some cases apoptosis, possibly due to loss of cell-cell contacts (118-120). N-cadherin interactions between tumor and vascular endothelial cells appear to partly mediate tumor cell-endothelial attachment and extravasation (121, 122).

Claudin-2 has been shown to be increased in breast cancers that metastasise to the liver. Its ability to mediate tumor cell-hepatocyte interaction is thought to facilitate arrest in this organ (123, 124). A further study has reported downregulation of claudin 4, claudin 7 and γ-catenin in breast cell line metastases to liver, with altered γ-catenin cellular localization. Interestingly, claudin 7 was also expressed by macrophage like cells surrounding the liver metastases, and was re-expressed in large tumors, suggesting possible interaction of the microenvironment in promoting metastasis (125).

Breast cancer commonly metastasizes to brain, with a prevalence of approximately 30% at autopsy (126, 127). Risk factors include high grade and stage, young age, estrogen receptor negativity and HER2 overexpression (128, 129). Brain metastasis requires breach of the blood brain barrier (BBB), a unique non-fenestrated endothelial structure that prevents passage of large molecules and cells. Tight and adherens junctions are
integral to the barrier function of brain microvascular endothelial cells (BMECs) [reviewed in (130, 131)]. BMECs display higher transepithelial electrical resistance and lower solute permeability than other endothelial cells, while their tight junctions are more complex and passage of polar solutes via the paracellular pathway is greatly reduced. The basement membrane is relatively thicker, and the underlying astrocytes regulate flow across the barrier. Among the proteins implicated in susceptibility to brain metastasis, loss of claudins 3 and 5 is associated with increased leakiness of the BBB (132, 133). Stromal cell derived factor-1α (SDF-1 α) is a chemokine expressed by several organs including the CNS, and expression of its receptor CXCR4 in breast cancer cells may facilitate BBB penetration. SDF-1 α treatment increases permeability of BMEC monolayers to breast tumor cell invasion, activating the PI-3K/AKT signaling pathway and causing endothelial cell retraction (134). HER2/Neu has been shown to upregulate CXCR4 expression (135). Matrix metalloproteinases also play a role in breast to brain metastasis; with MMP-2 & -3 activity increased in vivo (136, 137); and that of MMP-1 & -9 increased in vitro (138).

Having discussed many of the functional neoplastic processes relevant to breast cancer progression involving intercellular junctions, we will now examine some of the many proteins and membrane domains that have been implicated in junction-based signalling in breast cancer. As an exhaustive examination of all implicated proteins and domains would be beyond the scope of this short review, we will focus on a select few and apologise to those authors whose work it was not possible to cover.

**Ion Channels, Tight Junctions and Breast Cancer**

The sodium potassium ATPase (Na⁺ K⁺ ATPase) ion channel has emerged as an important regulator of tight junction function. This heterodimeric protein consists of 3 subunits; α, β, and a member of the FXYD family (139-142). It primarily functions as an ion channel, exporting three sodium ions and importing two of potassium against their concentration gradients in an ATP-dependent reaction, maintaining transmembrane ion concentrations. Its activity is increased in lactating mammary gland and in response to prolactin in vitro (143, 144), suggesting a role in lactation.

Na⁺ K⁺ ATPase function plays roles in the establishment of cell polarity, assembly of tight junctions, and regulation of paracellular permeability. Rajasekeran et al demonstrated in various epithelial cell types that tight junction formation was reversibly inhibited by inhibition of Na⁺ K⁺ ATPase, either by depletion of K⁺ or by treatment with the inhibitor ouabain. Notably, this occurred only in the presence of Na⁺ containing media. Additionally they showed that not only adequate Na⁺ K⁺ ATPase pump function, but also expression of E-cadherin and Na⁺ K⁺ ATPase β subunit, are necessary for tight junction formation and normal polarization (145, 146). They therefore hypothesised that Na⁺ K⁺ ATPase and E-cadherin function synergistically in assembling tight junctions (147).
Interestingly, while treatment with high doses of ouabain causes Na\(^+\) K\(^+\) ATPase inhibition and alters cell-cell and cell-substrate contact, increasing permeability and decreasing trans epithelial resistance (145, 148); treatment with nanomolar doses that exhibit no significant effects on pump function decrease tight junction permeability to both ions and non ionic molecules, accompanied by alterations in expression of claudins 1, 2 and 4 (149). An endogenous form of ouabain, synthesized and stored in mammalian adrenal cortex and hypothalamus, has been suggested to act as a hormone (150), perhaps in part functioning to modulate tight junctions.

Ouabain and its related drugs, a class known as cardiac glycosides, have been suggested to have efficacy in the treatment of a variety of malignancies including breast cancer. Several epidemiological studies have suggested that breast cancer patients taking these drugs had better-differentiated tumors, exhibited less distal metastasis, had fewer recurrences and statistically lower disease-related mortality (151-153). It has been shown that cardiac glycosides induce anti-proliferative effects in breast cancer cells at concentrations too low to significantly affect Na\(^+\) K\(^+\) ATPase pump function (154). It is tempting to speculate that these effects may relate (at least in part) to anti-proliferative signals transmitted through the tight junctions whose formation is facilitated at these low glycoside concentrations. Furthermore, anti-cancer implications of glycoside-induced tight junction enhancement are also consistent with a nutritional model of cancer which proposes that tight junction leakiness facilitates nutrient and growth factor access to “nourish” the cores of solid tumors (155).

**Lipid Rafts and Adhesion Proteins**

While the classical model of the plasma membrane describes the membrane as a liquid-disordered phospholipid bilayer with molecules such as proteins and cholesterol randomly interspersed (156), this model is now viewed as an oversimplification. Specifically it has been recognised that cholesterol and several proteins involved in dynamic cellular processes are non-randomly segregated into liquid-ordered membrane microdomains known as lipid rafts, which form as a consequence of tight spatial packing of predominantly sphingolipids rather than phospholipids. This was initially postulated based on such observations as the ability of differing phases to co-exist in lipid bilayers (157, 158); differential distribution and clustering of membrane lipids (159-161); and the presence of sphingolipid-enriched detergent-resistant regions of cell membranes (162). Simons et al were among the first to define the concept of lipid rafts (163), which have been defined as “small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes.” (164).

Several adhesion proteins such as occludin, ZO-1 (165, 166), claudins 1, 3, 4, 5, 7, 8 (166-168) and JAM-A (168) have been described to be associated with lipid raft domains, and it has even been suggested that tight junctions themselves constitute a subtype of raft domain (165). The tyrosine kinases Src and EGFR both localize in lipid rafts (169-171); both regulate cadherin/catenin interactions (172, 173), and both have been strongly implicated in pro-malignant signaling in breast cancer (173, 174). In addition,
components of the Wnt signaling pathway may be lipid raft associated (175, 176). It has been suggested that cholesterol in lipid rafts stabilizes the protein complexes in tight junction strands (168).

While junctions play an important role in cell migration, it has also been shown that lipid rafts modulate front-rear polarity in migrating MCF 7 breast cancer cells (177), and work from our group has revealed shuttling of the hyaluronan receptor CD44 in and out of lipid raft domains of breast epithelial cells according to migratory status (178). Similarly, unpublished work from our group suggests shuttling of Na⁺ K⁺ ATPase in and out of raft domains in breast cancer cells lines, in a hormone receptor status-dependent manner, in response to treatment with anti-proliferative doses of cardiac glycosides. It is intriguing to speculate that similar changes may occur in breast cancer with raft-associated junctional proteins in response to such junction-dependent processes as cell migration.

Clinical Application of Adhesion Molecules in Breast Cancer

A number of tight and adherens junction proteins have been suggested or investigated as potential biomarkers in breast cancer. As discussed above, claudin expression has been closely examined, and the claudin-low subtype (defined by low gene expression of claudins 3, 4 and 7 and associated with an aggressive clinical picture (114)), thus claudin characterization will likely become a routine part of breast cancer diagnostic and prognostic workup.

P-cadherin has been identified as a cancer stem cell marker for basal-type breast cancer (94, 179), and has been shown to be an independent marker for disease-free, but not overall, survival (180). While the prominent role of E-cadherin downregulation in EMT would make it a tempting proposition as a prognostic indicator (and in fact E-cadherin loss has diagnostic value in lobular carcinomas), used alone its correlation with prognosis has been variable (181-186). One study found a reduction in one of E-cadherin, β-catenin, α-catenin and plakoglobin to correlate significantly with breast cancer metastasis (187), and a recent paper has indicated a combination of E-cadherin and carcinoembryonic antigen as a useful predictor of relapse (186). As E-cadherin sequestering of β-catenin in adherens junctions prevents it partaking in pro-neoplastic canonical Wnt signaling, it would be logical that a measure of β-catenin distribution might be of prognostic benefit. One study found that a novel scoring system of membrane minus cytoplasmic β-catenin correlated with worse outcome in breast cancer (188).

Despite the obvious theoretical promise of cell junctions and their proteins as anti-metastatic therapeutic targets, junction-directed therapies are still in their infancy. Perhaps the greatest potential lies with targeting Claudins 3 and 4, which have been recognised as the receptors for the permeability-enhancing lytic toxin Clostridium perfringens enterotoxin (CPE) (189). This may be a useful therapy in breast cancers overexpressing these proteins, as it has been shown to induce lysis of claudin 3 & 4 overexpressing breast cancer cell lines (190).
Another exciting target is JAM-A, following a recent publication demonstrating anti-proliferative efficacy of a function-blocking JAM-A antibody in xenograft murine models of breast cancer (191). Unpublished work from our group has also shown promising *in vitro* and pre-clinical *in vivo* efficacy of a novel small molecule inhibitor of JAM-A, which we speculate could be particularly valuable in aggressive breast cancers concomitantly over-expressing HER2 and JAM-A.

ADH-I, an anti-N cadherin protein, has shown efficacy against pancreatic and prostate cancer in preclinical studies (192, 193), in addition to promising effects on disease stabilisation in early clinical trials (194). However, it has yet to be evaluated in breast cancer.

While the targeting of junctional proteins in breast cancer is still in its infancy, the expanding roles of these proteins in driving malignant signaling processes suggest many exciting targets for future research.

**Conclusion**

It is clear that breast ductal adhesion complexes and their constituent proteins play vital roles in breast physiology and pathology, not just by exerting mechanical effects but also through influencing key cell signaling and gene transcription events. While modulation of the physical properties of breast ductal barriers is essential for cyclical changes in lactation and engorgement, we have also discussed the role of junctional proteins in changes such as ductal development in embryogenesis and puberty. We have further examined how alterations in junctional integrity can contribute to such conditions as breast inflammation and metastasis and invasion of breast cancer. We have discussed the role of junctional proteins in dysregulated signal transduction and gene transcription leading to neoplastic phenomena such as proliferation, dedifferentiation, invasion and metastasis. Finally, we have explored some of the many exciting prospects for junctional proteins as both prognostic biomarkers and as therapeutic targets. It is the latter function of junctional proteins that is currently the focus of much research, and that may yield meaningful contributions to patient care in the future.


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